STATUS AND FUNCTIONAL INDICATORS OF FOLATE AND CHOLINE METABOLISM AMONG THIRD-TRIMESTER PREGNANT, LACTATING, AND NONPREGNANT WOMEN: A SET OF DOSE RESPONSE STUDIES

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STATUS AND FUNCTIONAL INDICATORS OF FOLATE AND CHOLINE METABOLISM AMONG THIRD-TRIMESTER PREGNANT, LACTATING, AND NONPREGNANT WOMEN: A SET OF DOSE RESPONSE STUDIES

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ABSTRACT

Background: Folate and choline are essential nutrients of particular importance during pregnancy and lactation and among nonpregnant women of reproductive age. However, in the era of folic acid (FA) fortification, high folate status and supplement use during pregnancy have been linked to adverse outcomes including asthma and eczema in children. No dose response studies conducted with women informed the choline Adequate Intakes (AIs), meanwhile evidence suggests that intakes greater than the current AI could optimize pregnancy endpoints. Research informing intake recommendations and strategies that promote optimal outcomes while avoiding inadequate *and* excessive intakes of folate and choline are needed.

Objective: This research sought to quantify status and functional indicators of folate and choline metabolism in response to controlled intakes among pregnant, lactating, and nonpregnant women.

Design: Pregnant (*n*=26), lactating (*n*=28), and nonpregnant (*n*=21) women consumed: a prenatal supplement containing 750 µg FA; 480 or 930 mg choline/d ; and 200 mg docosahexaenoic acid (DHA)/d for 10–12 wks. Biological samples were collected throughout the study. Folate status indicators were measured in all groups.

Phosphatidylcholine(PC)-DHA, a functional indicator of choline metabolism, was assessed in pregnant and nonpregnant women.

Results: (1) At study-end, serum folate concentrations did not differ (P=0.876) by physiologic group and urinary folate excretion represented ~9–43% of intake. Breastmilk FA concentrations increased (P=0.003), while total folate remained constant (P= 0.244) over time.

(2) PC-DHA was greater (P=0.01–0.001) among pregnant women at baseline. PC-DHA increased (P<0.001) in both groups regardless of choline intake; however, among nonpregnant women, consumption of 930 mg choline/d led to greater (P=0.011) PC-DHA.

Conclusions: (1) The study folate dose yielded supranutritional folate status in all physiologic groups. Given unresolved concerns about exposure to excess FA and the widespread folate adequacy of our FA-fortified population, it may be prudent to reduce the amount of FA in prenatal supplements.

(2) Via its capacity to donate methyl groups, a higher choline intake may facilitate synthesis and delivery of DHA to peripheral tissues among nonpregnant women and increase availability of methyl groups during pregnancy. These beneficial endpoints of choline intakes greater than the current AIs warrant further exploration.

BIOGRAPHICAL SKETCH

Allyson A. West grew up in rural northwest Illinois. She was valedictorian of the Morrison High School Class of 1999. In 2002 she earned a culinary degree at the Cooking and Hospitality Institute of Chicago. After a stint at the renowned Chicago restaurant Charlie Trotter's, Allyson returned to school to pursue her undergraduate degree in Food Science and Human Nutrition at the University of Illinois Urbana-Champaign. She graduated with her BS degree in 2007, earning the Bronze Tablet distinction and being awarded Human Nutrition student of the year. In the fall of 2008, Allyson matriculated to the graduate program in the Division of Nutritional Science at Cornell University. While at Cornell, she was a teaching assistant for two semesters and was later granted an American Egg Board – Egg Nutrition Center Dissertation Fellowship. Allyson completed her dissertation research under Dr. Marie A. Caudill studying folate and choline metabolism and requirements among pregnant, lactating, and nonpregnant women. Upon completion of her PhD, Allyson plans to embark on a teaching career in the Boston, MA area.

Dedicated to Dr. Yovanni A. Cataño-Lopera

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Making it to this point has only been possible with the generous assistance and support of many people.

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5-formyl-THF:	5-formyltetrahydrofolate
5-methyl-THF:	5-methyltetrahydrofolate
5-methyl-THFolate:	5-methyltetrahydrofolate
AI:	Adequate Intake
ALT:	alanine aminotransferase
ARA:	arachidonic acid
BHMT:	betaine-homocysteine *S*-methyltransferase
Cob(I):	cob(I)alamin
Cob(II):	cob(II)alamin
Cob(III):	methylcob(III)alamin
CV:	coefficient of variance
DPA:	docosapentaenoic acid
DFE:	Dietary Folate Equivalent
DHA:	docosahexaenoic acid
DHF:	dihydrofolate
DHFR:	dihydrofolate reductase
DMG:	dimethylglycine
DRI:	Dietary Reference Intake
DTA:	docosatetraenoic acid
dUMP:	deoxyuridine monophosphate
EPA:	eicosapentaenoic acid
FA:	folic acid
HMRU:	Francis A. Johnston and Charlotte M. Young Human Metabolic Research Unit
IOM:	Institute of Medicine
IS:	internal standard
LC-MS/MS:	liquid chromatography-tandem mass spectrometry
LMM:	linear mixed model
LoA:	Bland Altman Limits of Agreement

LOD:	limit of detection
LOQ:	limit of quantification
MA:	microbiological assay
MT:	generic methyltransferase
MTHFD1:	methylenetetrahydrofolate dehydrogenase
MTHFR:	methylenetetrahydrofolate reductase
MTR:	methionine synthase
MTRR:	methionine synthase reductase
NAFLD:	non-alcoholic fatty liver disease
NHANES:	National Health and Nutrition Examination Survey
NIST:	National Institute for Standard and Technology
NTD:	neural tube birth defect
PC:	phosphatidylcholine
PE:	phosphatidylethanolamine
PEMT:	phosphatidylethanolamine *N*-methyltransferase
PtEth:	phosphatidylethanolamine
PtCho:	phosphatidylcholine
RBC:	red blood cell
RDA:	Recommended Daily Allowance
RFC:	reduced folate carrier
SAH:	*S*-adenosylhomocysteine
SAM:	*S*-adenosylmethionine
SAX:	strong anion exchange
SHMT:	serine hydroxymethyltransferase
SPE:	solid phase extraction
UL:	Tolerable Upper Intake Level
THF:	tetrahydrofolate
TS:	thymidylate synthase

PREFACE

Background

Folate and choline are essential nutrients related to one another through one-carbon metabolism (Figure P.1). Adequate folate and choline nutrition are protective of neural tube birth defects (NTDs) that form early in pregnancy (1,2); provide one-carbon units for essential methyltransferae reactions; and are required for cell division (folate as a DNA precursor and choline as a component of phosphatidylcholine (PC), the membrane forming phospholipid). Thus, folate and choline nutriture are of particular importance during pregnancy and lactation and among nonpregnant women of reproductive age who may become pregnant.

Folate Metabolism

Folate refers to a group of related water soluble molecules with essential roles in nucleotide (purines and thymidylate) biosynthesis and amino acid metabolism. The primary form of folate in circulation is the reduced 5-methyltetrahydrofolate. Folic acid (FA) is a synthetic fully oxidized form of folate added to fortified foods and used in dietary supplements. Many forms of folate are enzymatically inter-convertible, although FA must be reduced to tetrahydrofolate in order to be utilized by the body (Figure 1)

Folate Dietary Reference Intakes (DRIs)

The Recommended Daily Allowance (RDA) is an intake value set to satisfy the nutritional needs of ~97% of individuals in a gender and life stage group. The Tolerable Upper Intake Level (UL) is a level above which there is increased risk of adverse effects. The folate RDAs are expressed as Dietary Folate Equivalents (DFEs)

to account for the greater bioavailability of FA versus naturally occurring food folate. The current folate RDA values for nonpregnant, pregnant, and lactating women are 400, 600, and 500 µg DFEs/day, respectively (3). The folate RDAs for nonpregnant and pregnant women are based on intakes that maintain blood folate levels above adequacy cut-offs, while the RDA for lactating women adds the amount of folate lost through breastmilk to the RDA for nonpregnant women (3). The UL is 1000 µg FA/day which is related to the potential for excess folate intake to mask vitamin B12 deficiency (3). Importantly, folate dose response studies have not been conducted with third-trimester pregnant or lactating women.

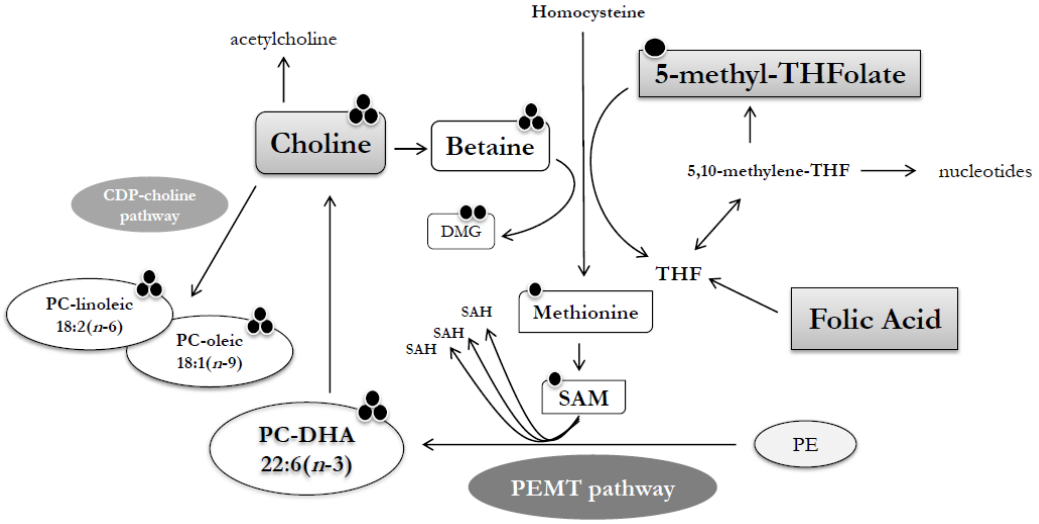

Figure P.1 Folate and choline in one-carbon metabolism

One-carbon methyl units (denoted by black dots) from 5-methyl-THFolate and choline, via betaine, are used to methylate homocysteine to methionine. Methionine is converted to S-adenosylmethionine, which participates in methylation reactions, including the methylation of PE to PC via the PEMT pathway.

Abbreviations: 5-methyl-THFolate, 5-methyltetrahydrofolate; DMG, dimethylglycine; PE, phosphatidylethanolamine; PEMT, phosphatidylethanolamine *N*-methyltransferase; PC, phosphatidylcholine; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; THF, tetrahydrofolate

Folic acid exposure and neural tube defects

Pre-conception FA supplementation reduces incidence of NTDs (1,4). The Centers for Disease Control and Prevention and Institute of Medicine (IOM) recommend that women of childbearing age consume 400 μg FA/day in addition to food folate from a healthy diet for the prevention of NTDs (3,5). To help women achieve FA intake goals and reduce NTD incidence, federally mandated FA fortification of enriched cereal grain products designed to deliver ~100 μg FA/day was implemented in 1998 (6). In addition to FA added to the food supply (i.e., fortified foods), the other important source of FA exposure is dietary supplements. Although FA-containing supplement use varies widely by ethnicity, education, and age, studies show ~38% of adult females and ≥80% of pregnant and lactating women, consume multivitamin supplements (7–9). Prenatal multivitamin supplements are produced specifically for and marketed to women of childbearing age and are generally labeled to contain 800–1000 μg FA, an amount that exceeds RDAs for all physiologic groups.

Folate status in the era of folic acid fortification

Since FA fortification was implemented, population wide folate status has improved dramatically: National Health and Nutrition Examination Survey (NHANES) data show that mean serum folate in females increased from 5.8 ng/mL pre-fortification to 14.1 ng/mL post-fortification (10). Improved folate status among women of reproductive age has reduced NTD incidence in the United States by19–60% (11,12); however, putative effects of increased FA exposure have become an issue (13). High or supranutritional folate status, which is typically the result of FA supplement use (14), has been linked to cognitive impairment via high folate/low

vitamin B12 imbalance and a purported “dual role” in cancer prevention and promotion (13). What is more, an emerging body of literature suggests *in utero* exposure to maternal supranutritional status and/or FA-containing supplement use is associated with adverse outcomes in children such as increased risk for asthma and eczema (15,16).

The 1998 folate DRIs were based on studies executed before the advent of FA fortification and the accompanying increase in population wide folate status. In addition, high prevalence of FA-containing supplement use highlights this source of FA exposure among pregnant and lactating women. In order to refine folate intake guidelines going forward, controlled feeding studies measuring folate status that results from the consumption of a realistically achievable folate intake (e.g., folate from a mixed diet and over-the-counter FA-containing prenatal supplement) within the context of population wide folate sufficiency are warranted.

Choline metabolism

The essential micronutrient choline may be: (1) a source of one-carbon units via its oxidative product betaine; (2) a precursor of PC, the ubiquitous phospholipid; and/or (3) used to generate the neurotransmitter acetylcholine (Figure 1). The phosphatidylethanolamine *N*-methyltransferase (PEMT) pathway generates PC via the addition of three methyl units to the phosphatidylethanolamine (PE) molecule; thus PEMT is a PC biosynthetic pathway as well as the source of de novo choline synthesis in the body as free choline may be hydrolyzed from the PC molecule (Figure 1).

Choline Adequate Intakes

The IOM publishes Adequate Intakes (AIs) when there is not enough evidence to establish RDAs. Although plasma choline decreases given a choline deficient diet, under normal conditions plasma choline is not well correlated with choline intake, in addition, folate, vitamin B12, and methionine status may impact plasma choline concentrations (3,17). Consequently, circulating levels of the liver enzyme alanine aminotransferase (ALT), an indicator of liver dysfunction, was used in setting the AI. Dose response studies conducted with women were not available for consideration when the choline AIs were published in 1998. Thus, the 425 mg choline/day AI value for nonpregnant women was based upon the amount required to prevent elevation of ALT in adult males (3). The 450 mg choline/day AI value for pregnant women added an increment of 25 mg choline/day to the nonpregnant AI to account for choline accretion of the fetus and placenta; this value was extrapolated predominately from animal data (3).

Human and animal research suggests that there is increased demand for choline during pregnancy due to maternal metabolic needs associated with gestation as well as fetal development and accumulation (18). In addition, studies in animals show that the offspring of choline supplemented mothers have improved memory and protection against age related cognitive decline (19). Taken together this body of evidence indicates that the current AI for pregnant women, 450 mg choline/day, may not optimize outcomes for mothers or their babies.

Choline and lipid metabolism

Choline metabolism is central to hepatic lipid metabolism as PC is required for lipoprotein formation and the export of triglycerides from liver. PC is composed of a three carbon chain linked to a phosphocholine head group and two fatty acids. Choline is a substrate of the CDP-choline pathway which synthesizes PC containing the unsaturated linoleic and oleic fatty acids (20) (Figure 1). In addition, methyl groups from the choline metabolite betaine may be used to generate PC via the PEMT pathway which produces PC enriched with polyunsaturated fatty acids (Figure 1). Notably, PC enriched with docosahexaenoic acid (DHA), a long chain polyunsaturated fatty acid with essential roles in human health throughout life , appears to be exclusively synthesized via PEMT (20). The use of plasma PC-DHA has been suggested as a proxy for assessing hepatic PEMT activity (21); however, it is unclear how a greater choline and/or DHA intake may influence flux through the PEMT pathway.

PC is one of several phospholipids that make up cellular membranes and are used to form hepatic lipoproteins. Another important phospholipid is PE, which is also the precursor of PC formed through the PEMT pathway. Maintenance of the PC:PE ratio in cellular membranes has profound effects on membrane functionality (22,23); thus it is of interest to ascertain whether the increased demand for choline during pregnancy effects this ratio, and whether choline intake among pregnant and nonpregnant women influences erythrocyte and/or plasma PC:PE.

Functional indicators of choline metabolism

The use of elevated liver enzymes, such as ALT, are useful indicator s of choline metabolism and status under conditions of choline deficiency; however, functional indicators of choline metabolism and status among healthy populations are still needed. It is clear that choline/betaine intake and DHA metabolism are linked through the PEMT PC biosynthetic pathway; perturbations in the relationship between methyl donors and polyunsaturated fatty acid metabolism have been noted in cystic fibrosis (24) and Alzheimer's disease (25). In addition, the ratio of PC:PE also reflects choline metabolism as reductions in membrane and plasma PC:PE ratios have been associated with choline deficiency and impaired one-carbon metabolism (27,28). Nonetheless, how choline intake impacts erythrocyte and plasma PC-DHA and PC:PE ratios, i.e. functional indicators of choline metabolism, in healthy pregnant and nonpregnant women remains to be elucidated.

The Caudill Lab

Research in the Caudill lab group investigates nutrient metabolism and requirement with the long term goal of optimizing intake recommendations. To this end, the Caudill lab conducted a12-week controlled feeding study with third-trimester pregnant women, lactating women 5–15 weeks postpartum, and nonpregnant women from January 2009 through December 2010 designed to answer pressing questions regarding folate and choline nutrition in these populations. The overarching aims of this study were:

1. To elucidate how pregnancy and lactation influence folate and choline metabolism.
2. To determine whether a choline intake greater than the current AI confers benefit to third trimester pregnant women and their babies, lactating women and their babies, and/or nonpregnant women of childbearing age.

Within these broad aims, my dissertation research consisted of two projects with the following specific aims and hypotheses:

Project 1: *Folate status response to a controlled folate intake among nonpregnant, pregnant, and lactating women*

Specific Aim: To quantify folate status response to a known dose of folate comprised of a prenatal supplement containing 750 μg FA plus natural food folate (~400 μg DFEs) in third-trimester pregnant women, lactating women 5–15 weeks postpartum, and nonpregnant women of childbearing age.

Hypotheses:

1. Mean serum folate concentrations will exceed the "high" folate status cut-off in all groups.
2. Demand for folate will be greater in third-trimester pregnant and lactating women as evidenced by lower serum or RBC folate concentrations and/or lower urinary folate excretion.

Significance and Innovation

With implications for fetal development (with respect to NTDs) as well as healthy aging (with respect to cancer risk), proper folate nutrition is essential for optimal health. This study is significant because it will inform future folate intake guidelines by longitudinally assessing folate status among third-trimester pregnant, lactating, and nonpregnant women consuming a readily generalizable folate intake i.e., folate from a mixed diet and over-the-counter FA-containing prenatal supplement.

This study is innovative because it will be the first that includes participants representing all three physiologic stages, i.e. non-pregnancy, pregnancy, and lactation, consuming an identical folate dose, which allows straight forward comparison of folate status, utilization, and requirement.

Project 2: *The influence of choline intake and pregnancy on phosphatidylcholine docosahexaenoic acid enrichment among third-trimester pregnant and nonpregnant women*

Specific Aims: (1) To determine whether choline intake influences erythrocyte and plasma PC-DHA and the PC:PE ratio among third-trimester pregnant and nonpregnant

women, and (2) To characterize the impact of pregnancy on erythrocyte and plasma PC fatty acid constituents and PC:PE.

Hypotheses:

1. A higher choline intake will increase PC-DHA in erythrocytes and plasma.
2. A higher choline intake will increase the PC:PE ratio in erythrocytes and plasma.
3. Erythrocyte and plasma fatty acids constituents and the PC:PE ratio will differ between pregnant and nonpregnant women, with greater PC-DHA concentrations and lower PC:PE ratio found among pregnant women.

Significance and Innovation

The study of choline metabolism as it relates to maternal and fetal outcomes is an emerging field. Dose response studies conducted with women of reproductive age are necessary for the refinement of choline intake recommendations that optimize the health of women and their children. This study is significant because it will inform future choline intake recommendations for understudied populations, i.e. pregnant and nonpregnant women, by quantifying response in third-trimester pregnant and nonpregnant women consuming two choline intakes: 480 mg choline/day, an amount approximating the current choline AI for pregnant women; and 930 mg choline/day, an amount roughly double the current AI for pregnant women.

There is not a strong relationship between choline intake and plasma choline levels, thus other functional indicators of choline metabolism, utilization, and requirement are needed. This study is innovative because it explores the use of

erythrocyte and plasma PC-DHA and PC:PE ratio as functional indicators of choline metabolism in a population of great interest, women of reproductive age.

Summary

These projects ask questions that will provide information for refining folate and choline intake recommendations and guidelines as well as provide insight into folate and choline metabolism among pregnant, lactating, and nonpregnant women. This work yielded two original research manuscripts (Chapters 1 & 2) and one methodological short communication (Chapter 3); in addition, I authored a review paper on the implications of genetic variation on folate and choline bioefficacy (Appendix A). Please enjoy the fruits of my labor!

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CHAPTER 1

Folate status response to a controlled folate intake among nonpregnant, pregnant, and lactating women[1]

ABSTRACT

Background: Folate dose response studies with women of childbearing age consuming a folic acid (FA)-containing multivitamin in the era of FA fortification are lacking.

Objective: We sought to investigate folate status response to a known folate dose comprised of a FA-containing prenatal supplement plus natural food folate among third-trimester pregnant women, lactating women 5–15 wks postpartum, and nonpregnant women.

Design: Pregnant (n = 26), lactating (n = 28), and nonpregnant (n = 21) women consumed the study folate dose under controlled intake conditions for 10–12 wks. Blood, urine, and breastmilk were collected at baseline, study-midpoint, and study-end.

Results: Study-end serum total folate concentrations averaged ~30 ng/mL and did not differ by physiologic group (P= 0.876). Study-end urinary folate excretion represented ~9–43% of total folate intake and ranged from 100–500 µg/day. Third-trimester pregnant women excreted less urinary folate than lactating (P= 0.075) and nonpregnant (P< 0.001) women. Lactating women excreted less (P< 0.001) urinary

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FA than nonpregnant women. Breastmilk total folate concentrations remained constant (*P*= 0.244; 61.8 ng/mL at study-end), while breastmilk FA concentrations increased (*P*= 0.003) to 24.1 ng/mL at study-end.

Conclusions: Consumption of the study folate dose yielded supranutritional folate status regardless of physiologic state. Based on urinary folate, folate requirements were: pregnant > lactating > nonpregnant women. Breastmilk folate species were responsive to maternal folate intake with FA making up ~40% of breastmilk total folate at study-end. These findings warrant revisiting prenatal supplement FA formulation in populations exposed to FA fortification programs.

INTRODUCTION

Folate refers to group of related molecules with essential roles in cellular methylation, nucleotide biosynthesis, and amino acid metabolism. Folic acid (FA) is a synthetic oxidized form of folate added to fortified foods and used in dietary supplements. In contrast, the majority of natural food folate is reduced, has a polyglutamate tail, and is ~40% less bioavailable than FA. In the U.S., folate requirements are met through natural food folate, FA-fortified foods, and folate/FA-containing vitamin supplements (1). To account for differences in bioavailability, folate recommended intakes are given in dietary folate equivalents (DFEs) where 1 µg natural food folate and 1 µg FA are equivalent to 1 DFE and 1.7 DFEs, respectively (2).

Pregnancy and lactation increase the demand for folate; current RDAs for nonpregnant, pregnant, and lactating women are 400, 600, and 500 DFEs/day, respectively (2). The Institute of Medicine and Centers for Disease Control and Prevention also recommend that women of child-bearing age consume 400 µg FA/day in order to decrease the incidence of neural tube birth defects (NTDs) (2,3). In 1996 the Food and Drug Administration mandated that enriched grain products be fortified with FA to facilitate achievement of this recommended intake (4). As a result of FA fortification, folate status among women of childbearing age has dramatically improved (5) and incidence of NTDs has decreased 19–50% (6,7).

Multivitamins are the most common dietary supplement consumed in the U.S (8). Most multivitamins contain 400 µg FA; however, prenatal multivitamin supplements often contain 800–1000 µg FA (9). Women of childbearing age are

advised to consume 400 μg FA per day through a combination of FA-fortified food and supplements (2); however, no explicit public health recommendation for healthy pregnant or lactating women to consume FA-containing supplements currently exists (10).

NHANES 2003–2006 data and Gallup polling for the March of Dimes indicate 30–38% of women of childbearing age consume FA-containing supplements/multivitamins (8,11). Studies have found that 77–93% of pregnant (12–14) and 73% of lactating (14) women use multivitamins; however, accurate prevalence is unknown among lactating women as this estimate does not come from a nationally representative study (10).

Folate dose response studies are lacking in third-trimester pregnant and lactating women. In addition, there has not been a comprehensive longitudinal assessment of folate status achieved by women of childbearing age consuming an over-the-counter FA-containing prenatal supplement in the era of FA fortification. This study sought to quantify folate status response to a known dose of folate comprised of a FA-containing prenatal supplement plus natural food folate (~400 μg DFEs) in third-trimester pregnant women, lactating women 5–15 weeks postpartum, and nonpregnant women of childbearing age.

STUDY PARTICIPANTS AND METHODS

Study Participants

Nonpregnant, third trimester pregnant (27 weeks gestation), and lactating women 4 weeks postpartum aged ≥ 21 y were recruited from the Ithaca, NY area from January 2009–October 2010 as described by Yan et al. (15). During the screening stage, interested women provided a blood sample for blood chemistry profiling and a complete blood count. In addition, these women completed a health history and demographics questionnaire (see Appendix B for questionnaires). Important inclusion criteria for all participants were: (i) general healthiness as determined by the questionnaire, blood chemistry profile, and complete blood count; (ii) no drug or alcohol use; (iii) normal kidney and liver function; and (iv) willingness to comply with study protocol, i.e., eating a certain number of meals at the on-site location and not consuming food or beverages outside what was provided by the study. Additional inclusion criteria for pregnant women included: singleton pregnancy and no pregnancy associated complications, e.g., preeclampsia, gestational diabetes. Additional criterion for lactating women was the intention to exclusively breastfeed for the duration of the study. Exclusion criteria included: inability to comply with study protocols and use of prescription medications known to affect liver function. Eligible pregnant women were admitted to the study on a rolling basis at 26–29 weeks gestation; eligible lactating women were admitted to the study on a rolling basis at the start of 5 weeks postpartum; and eligible nonpregnant women were added as scheduling and space constraints allowed until the desired number of participants completed the study.

The study protocol was reviewed and approved by the Institutional Review Board for Human Study Participant Use at Cornell University and at the Cayuga Medical Center (the hospital where pregnant participants delivered their babies; Ithaca, NY). Study participants were compensated for participation and informed consent was obtained from all participants prior to their entry into the study (see Appendix B for informed consent documents).

Study Design, Diet, and Supplements

Design

This study was an extension of a controlled feeding study conducted in pregnant, lactating, and nonpregnant women randomized to 480 or 930 mg choline/day for 10–12 weeks (15). Throughout the controlled feeding period (10–12 weeks), all participants consumed an over-the-counter prenatal multivitamin supplement containing 750 µg FA daily, plus natural food folate (~400 µg) from a mixed diet. Nonpregnant and pregnant women participated in the controlled feeding study for 12 weeks, whereas, lactating women participated in the study for 10 weeks. Biological samples including blood, urine, and breastmilk were collected at baseline, study-midpoint (week 6), and study-end (week 10/12).

Diet

The study diet, as previously described (15), provided ~2000 kcal/day; however, participants could individualize their caloric intake by choosing to consume more or less of menu items and foods containing < 5µg folate per serving, such as unenriched rice, select juices and sodas, chips, and pudding (see Appendix B for study diet). Nonpregnant and pregnant women consumed meals on at least 5 days/week of

their choosing in the Francis A. Johnston and Charlotte M. Young Human Metabolic Research Unit (HMRU) at Cornell University. Lactating women consumed meals on at least 3 d/week of their choosing on-site in the HMRU. All other food and beverages were provided as take-aways.

To control FA intake, commercial items manufactured without FA (e.g., whole wheat bread and organic specialty pasta) were utilized and menu items prepared in the HMRU (e.g., pizza dough and muffins) used unenriched wheat flour. Thus, dietary folate intake was restricted to naturally occurring forms with the only source of FA being the study prenatal supplement.

Supplements

Each day study participants consumed an over-the-counter prenatal supplement (Pregnancy Plus®, Fairhaven Health, LLC, Bellingham, WA). Although this prenatal supplement was labeled to contain 600 μg FA per tablet, the measured amount was 750 μg FA (see analytic measurements for details on methodology) and is the value used throughout this manuscript. To achieve recommended nutrient intake levels not met with the study diet, study participants consumed also consumed a daily 200 mg docosahexaenoic (DHA) supplement (Neuromins, Nature's Way Products, Springville, Utah) (16) and a thrice weekly potassium/magnesium supplement (General Nutrition Corp., Pittsburgh, PA) . When eating on-site, participants consumed supplements under the supervision of study personnel. For days when study participants did not eat on-site, supplements were provided in baggies along with their take-away meals. Participants were instructed to consume the supplements with a meal of their choice.

Compliance

The study protocol was well-tolerated with 91% of enrolled participants completing the study (21 of 22 nonpregnant, 26 of 29 pregnant, and 28 of 31 lactating women). Reasons for stopping the study included nausea, early delivery, personal challenges, and food dislikes (15).

Study participants completed daily checklists indicating they received and consumed all menu items and supplements. For meals consumed onsite, study personnel were able to directly monitor compliance. For meals consumed off-site, participants were asked to return all empty baggies and take-away food containers to study personnel during their next visit to the HMRU. In addition, study personnel had daily contact with participants throughout the study to maintain positive rapport and enhance compliance.

Sample Collection and Processing

Blood: Fasting venous blood was drawn at baseline (all participants), study week 6 (all participants), study week 10 (all participants), and study week 12 (nonpregnant and pregnant participants) in the HMRU ward by a trained phlebotomist. Blood samples were collected in EDTA and serum separator tubes, processed within 2 hours, and stored in cryostat tubes at -80 ^{o}C until analysis as previously described (15). Ascorbic acid (10 g/L) was added to whole blood and serum samples during processing to preserve labile folates.

Urine: Participants completed 24-hour urine collections at baseline (all participants) and study weeks 6 (all participants), 9 (nonpregnant and pregnant), 10 (lactating), and 12 (nonpregnant and pregnant) in acid-washed opaque 2 L bottles as

previously described (15). To preserve labile urinary folates, 5 g sodium ascorbate was added to each bottle ahead of collection.

Breastmilk: Breastmilk samples were collected at baseline and study weeks 6, 9, and 10 with a Medella® electric breast pump in the HMRU. Lactating women were fasted for breastmilk sample collection which occurred on the same morning as the corresponding week's blood collection. Breastmilk samples consisted of the full expression of one breast 2 hours after the first feed of the day. Women expressed the same breast throughout the study. Upon collection, the breastmilk samples were immediately placed on ice, dispensed within 30 minutes of collection into 4.5 mL cryostat vials (Cryo; NUNC, Roskilde, Denmark), and stored at -80^{o}C until analysis.

Analytic Measurements

Food and breastmilk folate extracts

Natural food folate provided by the study menu (dietary folate) and breastmilk folate concentrations were quantified from extracts prepared using the trienzyme method. Blank extracts were prepared from extraction buffer subjected to trienzyme digestion in order to quantify folate in enzyme preparations. All incubations were performed at 37 ^{o}C.

For dietary folate extraction, prepared study meals were homogenized with cold 0.1 M potassium phosphate buffer with 57 mM ascorbic acid and stored at -80 ^{o}C. Upon thawing, meal samples (2 g) were mixed with extraction buffer (0.05 M CHES, 0.05 M HEPES, 0.1 M sodium ascorbate, 0.2 M 2-mercaptoethanol) and subjected to trienzyme digestion as described by Tamura et al. (17). After digestion, sample

extracts were filtered through Whatman paper, and stored at -80 ^{o}C until quantification.

Breastmilk sample extractions were prepared by the method of Lim et al. (18) with the following modifications. First, samples were mixed with 10 mg/mL sodium ascorbate and extraction buffer upon thawing. Second, after protease inactivation, pH was adjusted to 7.2 and samples were incubated for 5 hours with rat plasma conjugase (500 μL/mL breastmilk). Finally, following conjugase incubation, samples were centrifuged at 4^{o}C, filtered with 0.45 μm syringe filters (MillexHV), and stored at -80 ^{o}C. Breastmilk extracts for liquid chromatography-tandem mass spectrometry (LC-MS/MS) and microbiological assay (MA) quantification were prepared separately. A stable-isotope internal standard (IS) mix was added to breastmilk before enzyme digestion for samples destined for LC-MS/MS quantification. Extracts for LC-MS/MS and MA methods were prepared from 4 mL and 0.1 mL breastmilk, respectively

Microbiological assay measurements

The MA method (19) with *Lactobacillus casei* (ATCC 7469) as the test organism and10 ng/mL FA (Sigma) as the standard calibrator was used to determine total folate concentrations in serum, whole blood, urine, breastmilk, and dietary folate. The coefficients of variance (CVs) were calculated using a positive quality control sample of each analyte, i.e. repeated measurement of a sample with the same matrix as the study biological samples being measured. The purpose of the positive quality control sample was to ensure that the assay and calibrators were working correctly and to determine the precision of the assay. MA intra-assay CVs were < 10%. MA inter-

assay CVs were as follows: serum, whole blood, and breastmilk, < 10%; urine and dietary folate, < 13%.

Red blood cell (RBC) folate values for nonpregnant and pregnant women were calculated from whole blood folate concentrations, serum total folate concentrations, and hematocrit values. Lactating serum folate was not measured via MA; serum 5-methyl-THF + FA measured via LC-MS/MS was used a proxy for serum total folate.

Dietary folate was determined twice, the first using meal extracts prepared before the start of the study and the second using meal extracts prepared during the study. Dietary and breastmilk folate values were corrected for exogenous folate (i.e. folate from amylase and/or conjugase preparations added during trienzyme digestion) by subtracting blank extracts MA values.

LC-MS/MS measurements

5-methyltetrahydrofolate (5-methyl-THF) and FA were quantified in serum, urine, and breastmilk using liquid chromatography-tandem mass spectrometry (LC-MS/MS) stable-isotope dilution methods. Serum, urinary, and breastmilk extracts were prepared using solid phase extraction (SPE) clean-up methods on a 12-port vacuum manifold (J.T. Baker, Inc.). Extract eluents were concentrated with a SpeedVac centrifuge under vacuum with no heat before injection.

Standard curves were prepared from 5-methyl-THF (Sigma) and FA (Sigma) stock solutions as described in Pfeiffer et al. (20). National Institute for Standard and Technology (NIST) standard reference materials, SRM 3280 (multivitamin tablets) and SRM 1955 (homocysteine and folate in frozen human serum), were used to validate the accuracy of FA and 5-methyl-THF calibrator curves, respectively. Internal

standard (IS) solutions were prepared using ^{13}C-5-methyl-THF and ^{13}C-FA (Merck Eprova, Switzerland) as described in Pfeiffer et al. (20). CVs were calculated using positive quality control samples included with each extraction batch. Extraction batches included all time points (i.e. baseline, week 6, and study-end) for 4–10 participants. Details specific to the extraction method for each biological material are detailed below.

Serum: 5-methyl-THF and FA were extracted from 400 µL of serum using the SPE clean-up method of Pfeiffer et al. (20). Serum 5-methyl-THF and FA were quantified via LC-MS/MS (20) with modifications based on our instrumentation. The LC-MS/MS system used to quantify serum folates was a TSQ Quantum mass spectrometer (Thermo, San Jose, CA) equipped with a refrigerated Accela autosampler (Thermo) and Accela pump with degasser (Thermo). 5-methyl-THF and FA were separated with a Luna C18 column (250 x 4.6 mm, 5 µm bead size; Phenomenex) and matching guard column (4 x 3 mm) using a gradient mobile phase (500 µL/minute) consisting of solution A (10 mL/L acetic acid, 400 mL/L methanol, 100 mL/L acetonitrile) and solution B (0.15% formic acid). During the first 7 minutes of the run, a linear gradient brought the mobile phase from 50% to 100% solution A. This was followed by a linear gradient that returned the mobile phase to 50% solution A at minute 12. From minute 12 to minute 15 the column was re-equilibrated under isocratic conditions with 50% solution A. The retention times for 5-methyl-THF and FA were 5 and 9 minutes, respectively. The mass spectrometer was operated with electrospray ionization in positive ion mode with multiple reaction monitoring mode of the following transitions: 5-methy-THF, *m/z* 460→313; ^{13}C-5-methyl-THF, *m/z*

465→313; FA, *m/z* 442→295; ^{13}C-FA, 447→295. The total run time was 15 minutes, the injection volume was 15 µL, and the autosampler and column temperatures were set at 10°C and 25°C, respectively. Intra- and inter-assay CVs were 3.3 and 3.9%, respectively for serum 5-methyl-THF, and 8.5 and 13.7% for serum FA.

Due to the limited availability of ascorbic acid preserved serum (400–500 µL) and small amount of FA present, a limit of detection (LOD) and a limit of quantification (LOQ) were defined. Using serially diluted serum samples and a noise to signal ratio of 15 the serum FA LOD was set at 0.075 ng/mL. The intra-assay CV for samples < 0.11 ng/mL was 19.2%, whereas, the CV for samples with concentrations > 0.11 ng/mL was 8.5%; thus the serum FA LOQ was set at 0.11 ng/mL.

Urine: 5-methyl-THF and FA were extracted from 3 mL urine using an adaptation of the phenyl SPE method of Pfeiffer et al. (20). Briefly, urine samples were spiked with ^{13}C-5-methyl-THF and ^{13}C-FA IS preparation and mixed with 3 mL sample buffer (10 g/mL ammonium formate, 1 g/L ascorbic acid). Each sample was brought to pH 2.9 and allowed to equilibrate for 20 minutes at 20°C. Urines and calibrator points were cleaned up with 1 mL phenyl cartridges (100 mg; Bond Elut C18; Agilent) conditioned with 2 mL each of acetonitrile, methanol, and sample buffer (pH 2.9). After samples were loaded, cartridges were washed with 3 mL wash buffer (0.5 g/L ammonium formate, 0.05 g/L ascorbic acid; pH 3.4) and folates were eluted with 500 µL elution buffer (400 mL/L methanol, 100 mL/L acetonitrile, 10 mL/L acetic acid, 1 g/L ascorbic acid).

The LC-MS/MS Ion trap system used to quantify urinary folates consisted of a LCQ Advantage Max mass spectrometer (Thermo) equipped with a refrigerated Surveyor autosampler (Thermo) and Surveyor pump with degasser (Thermo). Urinary 5-methyl-THF and FA were separated using the same column and gradient system as serum folates described above. The retention times for 5-methyl-THF and FA were 5 and 8 minutes, respectively. The mass spectrometer was operated with electrospray ionization in negative ion mode with full scan parameters monitoring the following transitions: 5-methy-THF, *m/z* 458→329; ^{13}C-5-methyl-THF, *m/z* 463→329; FA, *m/z* 440→311; ^{13}C.FA, 445→311. The total run time was 15 minutes, the injection volume was 10 µL, and the autosampler and column temperatures were set at 10^{o}C and 25^{o}C, respectively. Intra- and inter-assay CVs were 7.3 and 10.1%, respectively for urinary 5-methyl-THF, and 10.4 and 11.6% for urinary FA.

Breastmilk: 5-methyl and FA were extracted from 4mL breastmilk using a strong anion exchange (SAX) SPE clean-up method based on those of Vishnumohan et al. (21) and Friesleben et al. (22). Briefly, trienzyme digested breastmilk extracts with ^{13}C-5-methyl-THF and ^{13}C-FA IS as described above were adjusted to pH 7.5 and cleaned up with 3 mL SAX cartridges (500 mg; Bond Elut SAX; Agilent) conditioned with 3 mL each of hexane, methanol, and MQ water; and 10 mL conditioning solution (0.01 M potassium phosphate, 0.1% ascorbic acid, 0.01% 2-mercaptoethanol; pH 7.5). After samples were loaded, cartridges were washed with 3 mL conditioning solution and folates were eluted with 1 mL elution buffer (5% sodium chloride, 1% sodium ascorbate, 0.1 M sodium acetate; pH 5.4).

Breastmilk folates were separated and quantified using the same Luna column and LC-MS/MS system as for serum folates described above. However, the mobile phase had to be modified in order to wash SAX elution buffer salts from the breastmilk extracts (22). Following 10 minutes of isocratic 7% solution A, a 3-minute linear gradient brought the mobile phase to 13% solution A. Then a 4-minute linear gradient brought the mobile phase to 30% solution A, followed by a second 4-minute linear gradient that brought the mobile phase to 90% solution A. A third 4-minute linear gradient brought the mobile phase back to 7% solution A. Finally, the column was re-equilibrated under isocratic conditions with 7% solution A for 10 minutes. The retention times for 5-methyl-THF and FA were 22 and 25 minutes, respectively, and the total run time was 35 minutes. Breastmilk folate concentrations were corrected for exogenous folate added during trienzyme digestion by subtracting blank extract LC-MS/MS values. Intra- and inter-assay CVs were 10.0 and 12.5%, respectively for breastmilk 5-methyl-THF, and 5.0 and 3.5% for breastmilk FA.

Prenatal supplement folic acid

The FA content of the prenatal supplement was determined using the LC-MS/MS negative ion mode method described in Nelson et al. (23). FA in the study prenatal supplement and NIST SRM 3280 multivitamin were extracted using the same protocol and quantified in the same LC-MS/MS run. The measured NIST 3280 FA concentration (412 mg FA / kg supplement) was consistent with the reference value published by Nelson et al. (413 mg/kg) (23).

MTHFR Genotyping

Methylenetetrahydrofolate reductase (MTHFR) C677T genotype was determined after purifying the PCR products (QIAquick PCR Purification kit, Qiagen) (24) and sequencing the double-stranded DNA templates with an Applied Biosystems Automated 3730 DNA analyzer.

Statistical Methods

Normality was not achievable in several dependent variable baseline concentrations, thus Kruskal-Wallis H- and Mann-Whitney U-tests were used to test baseline differences in folate status marker concentrations by physiologic group. Kruskal-Wallis H-tests were also used to test for differences in ethnicity/race, *MTHFR* C677T genotype, choline intake randomization, and supplement use prior to study enrollment distribution among the physiologic groups.

Linear mixed models (LMMs) were used to investigate folate status response over time when dependant variables and/or transformed dependant variables conformed to model assumptions. Log-transformed serum 5-methyl-THF, log-transformed serum 5-methyl-THF + FA, square-root transformed urinary 5-methyl-THF, square-root transformed urinary total folate, and log-transformed RBC folate were assessed using LMMs. Physiologic state (nonpregnant, pregnant, or lactating), time (study week), and choline intake level (480 or 930 mg/day) were entered as fixed factors and subject id was entered as random factor. Due to varying supplement use within and among physiologic groups before study entry, supplement use prior to study enrollment was entered as fixed factor. In addition, because the *MTHFR* C677T genotype can have robust effects on folate requirements (25, 26) and ethnicity may impact folate status (27), both were included as fixed factors in the LMMs. In the

initial model, all 2-way interactions between the fixed factors were included. Non-significant interactions were progressively removed until final models were derived. Bonferroni corrections were made for multiple comparisons where applicable. All breastmilk folate variables were square-root transformed and analyzed with LMMs as above without the physiologic group fixed factor.

To compare physiologic groups and assess response over time in variables that did not conform to LMM assumptions non-parametric analyses were used. Kruskal Wallis H- and Mann-Whitney U-tests were performed with baseline, week 6, and/or study-end concentrations as the test variable and physiologic state as the grouping variable. Wilcoxon rank sum tests stratified by physiologic state were utilized to assess change over time within and among physiologic groups. Serum FA and urinary FA were analyzed with non-parametric techniques. A portion of the serum FA values fell between the LOD and LOQ; concentrations for these samples were set equal to the LOQ (0.11 ng/mL) for statistical analyses.

All statistics were performed with IBM SPSS software (version 19; SPSS Inc, Chicago, IL).

RESULTS

Participant characteristics and baseline measurements

Seventy-five women were included in the final analyses. Twenty-one nonpregnant women completed 12 weeks of the study. Twenty-three pregnant women completed 12 weeks of the study and 3 completed 10 weeks of the study. Twenty-five lactating participants completed 10 weeks of the study, 2 completed 9 weeks, and 1 completed 8 weeks. The study-end time point was used in all statistical analyses and reflects the last sample collection for each study participant. The ethnicity/race of the study participants as well as *MTHFR* C677T genotype and choline supplement randomization were balanced among the physiologic groups (**Table 1.1**).

At baseline, 85, 75, and 33% of pregnant, lactating, and nonpregnant participants reported consuming FA-containing supplements ($P = 0.001$; Table 1.1). The differing rates of supplement use prior to study enrollment corresponded to differences in baseline folate status marker concentrations with pregnant and lactating participants having on average higher serum folate, greater urinary folate excretion, and higher RBC folate than nonpregnant participants (Table 1.1).

TABLE 1.1 Participant characteristics and baseline folate status marker concentrations for pregnant women ~27 wk gestation, lactating women ~5 wk postpartum, and nonpregnant women of childbearing age[1]

	Pregnant	Lactating	Nonpregnant	*P*-value
Number of subjects	26	28	21	
Ethnicity (Caucasian /African American / Hispanic / Asian / Other)	16 / 1 / 4 / 4 / 1	20 / 1 / 3 / 1 / 3	14 / 2 / 2 / 1/ 2	0.804
MTHFR genotype (CC / CT / TT)	11 / 13 / 2	11 / 15 / 2	9 / 9 / 3	0.974
Choline intake randomization (480 / 930 mg/day)	13 / 13	15 / 13	10 / 11	0.916
% using FA-containing supplement prior to study enrollment	85[a]	75[a]	33[b]	0.001
Serum folate (ng/mL)[2]				
5-methyl-THF	30.0 (24.4 – 34.7)[a]	31.4 (25.5 – 37.1)[a]	18.6 (14.7 – 23.7)[b]	<0.001
FA	0.41 (0.1 – 0.65)[ab]	0.54 (0.42 – 0.69)[b†]	0.32 (0.0 – 0.45)[a]	0.008
% with detectable FA	73[ab]	93[b†]	67[a]	0.062
5-methyl-THF + FA[3]	30.3 (24.9 – 35.6)[a]	33.7 (25.5 – 40.5)[a]	18.7 (15.2 – 23.7)[b]	<0.001
FA % of 5-methyl-THF + FA	1.5 (0.3 – 2.1)	1.9 (1.3 – 3.2)	1.5 (0.0 – 3.0)	0.222

Urinary folate excretion (μg / 24 hours)				
5-methyl-THF	144.0 (39.7 – 411.9)[a]	53.5 (14.2 – 165.6)[b]	5.3 (3.2 – 32.5)[c]	<0.001
FA[4]	5.6 (0.0 – 89.0)[a]	5.0 (0.0 – 18.4)[a]	0.0 (0.0 – 0.0)[b]	0.012
% with detectable FA	62[a]	57[a]	19[b]	0.008
Total folate	178.3 (84.0 – 475.9)[a]	113.4 (32.1 – 425.6)[a]	22.4 (10.0 – 65.8)[b]	<0.001
RBC folate (ng/mL)[2]				
Total folate	916.1 (735.7 – 1019.5)[a]	1295.8 (1095.6 – 1504.7)[b]	512.4 (459.5 – 572.1)[c]	<0.001

[1] Data were analyzed with Kruskal-Wallis tests; counts, percents, or medians (95% confidence interval) are presented; dissimilar superscripts within a row indicate Mann-Whitney test difference at $P < 0.05$

[2] To convert ng/mL to nmol/L folate, multiply ng/mL by 2.266

[3] Serum 5-methyl-THF + FA was used as a proxy for serum total folate due to the lack of serum MA measurement in lactating group

[4] Mean (standard deviation) urinary FA excretion (μg / 24 hours) at baseline was 62.5 (96.0) for pregnant women, 39.1 (67.8) for lactating women, and 11.2 (35.6) for nonpregnant women.

[†] Difference for pregnant group vs. lactating group, $P < 0.1$

Abbreviations used: 5-methyl-THF, 5-methyltetrahydrofolate; MTHFR, methylenetetrahydrofolate reductase; FA, folic acid; RBC, red blood cell

Folate dose

Average intake of natural food folate intake was 404 µg/day (~400 DFEs) (see Appendix B for folate in study diet). The FA content of the prenatal multivitamin supplement was 750 µg FA, yielding a total folate intake of 1150 µg/day (400 µg + 750 µg) or 1675 DFEs after adjusting for the 1.7x greater bioavailability of FA.

Serum Folate

Serum folate is a sensitive indicator of recent folate intake and can be a valid indicator of folate status (2). 5-methyl-THF is the primary form of folate found in circulation, while the presence and quantity of FA in fasted serum is of interest due to concerns regarding excess FA exposure (28).

Serum 5-methyl-THF

Consumption of the study folate dose (FA-containing prenatal supplement plus 400 µg natural food folate) yielded a small but significant increase (time, $P < 0.001$) in serum 5-methyl-THF over the course of the study that did not differ among the physiologic groups (physiologic group x time interaction, $P = 0.536$) after controlling for ethnicity, *MTHFR* C677T genotype, choline intake, and supplement use prior to study enrollment (**Figure 1.1A**). At study-end, serum 5-methyl-THF concentrations did not vary by physiologic group ($P = 0.855$; Figure 1.1A).

Serum folic acid

Consumption of the study folate dose did not alter serum FA concentrations within physiologic groups ($P = 0.122–0.302$) or among the cohort of participants as a whole ($P = 0.398$) over the course of the study (Figure 1.1B). At study-end, serum FA concentration did not vary by physiologic group ($P = 0.251$; Figure 1.1B).

Unmetabolized FA was present in fasted serum among 81%, 89%, and 81% of pregnant, lactating, and nonpregnant women, respectively, at study-end.

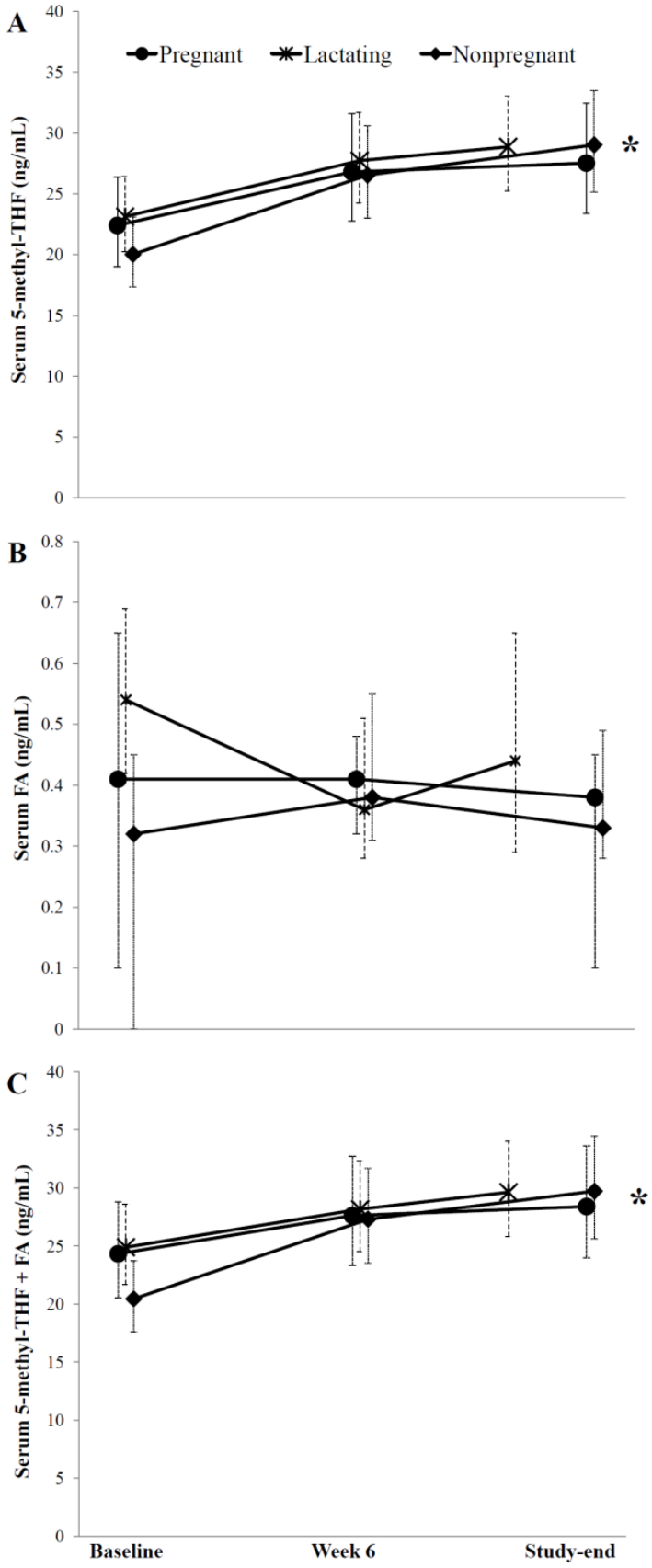
The percent of serum FA relative to serum 5-methyl-THF + FA, i.e. serum total folate, was small and did not vary over the course of the study within physiologic groups (P = 0.163–0.376). However, among the cohort of participants as a whole, FA percent of serum 5-methyl-THF + FA tended to decrease (P = 0.058) over the course of the study from 1.6% at baseline to 1.2% at study-end; FA percent of serum 5-methyl-THF + FA did not vary by physiologic group (P = 0.614) at study-end.

Serum 5-methyl-THF + folic acid

Similar to serum 5-methyl-THF, consumption of the study folate dose increased (time, $P < 0.001$) serum 5-methyl-THF + FA, i.e. total folate, over the course of the study that did not differ among the physiologic groups (physiologic group x time interaction, P = 0.275) after controlling for ethnicity, *MTHFR* C677T genotype, choline intake, and supplement use prior to study enrollment (Figure 1.1C). At study-end, serum 5-methyl-THF + FA concentrations did not vary by physiologic group (P = 0.893; Figure 1.1C). The study folate intake yielded study-end serum 5-methyl-THF + FA (i.e., total folate) concentration estimates exceeding 20 ng/mL (Figure 1.1C), an indicator of "high" folate status (5).

Figure 1.1 legend

Serum concentrations of 5-methyl-THF (A); FA (B); and 5-methyl-THF + FA (C) among third-trimester pregnant women (*n* = 26), lactating women 5–15 weeks postpartum (*n* = 28), and nonpregnant women of childbearing age (*n* = 21) consuming a FA-containing prenatal supplement plus 400 µg natural food folate for 10–12 weeks. Statistical analyses for 5-methyl-THF and 5-methyl-THF + FA were performed with linear mixed models. Statistical analyses for FA were performed with Kruskal-Wallis and Mann-Whitney tests. All participants were included in the analyses. Plotted data for 5-methyl-THF and 5-methyl-THF + FA are back transformed predicted means and 95% confidence intervals derived from linear mixed models; medians with 95% confidence intervals were plotted for FA. Study-end was week 10 for lactating participants and week 12 for pregnant and nonpregnant participants. Serum folate concentrations did not differ ($P = 0.251–0.876$) among physiologic groups at study-end. *indicates serum folate concentrations increased ($P < 0.001$) from baseline to study-end. Physiologic state did not interact with time for serum 5-methyl-THF and 5-methyl-THF + FA concentrations (P = 0.251–0.893); distribution of the serum FA data did not allow this interaction to be tested. To convert ng/mL to nmol/L folate, multiply ng/mL by 2.266. Abbreviations: 5-methyl-THF, 5-methyltetrahydrofolate; FA, folic acid


A
Pregnant
Lactating
Nonpregnant
Serum 5-methyl-THF (ng/mL)
40
35
30
25
20
15
10
5
0
*
B
Serum FA (ng/mL)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
C
Serum 5-methyl-THF + FA (ng/mL)
40
35
30
25
20
15
10
5
0
*
Baseline
Week 6
Study-end

Urinary Folate

Twenty-four hour urinary folate excretion is a useful folate status indicator because it reflects saturable intracellular retention and renal reabsorption mechanisms related to folate utilization and requirement (29). Further, in contrast to fasting serum folate which reflects folate status at its lowest point, 24-hour urinary folate excretion encompasses the rise and fall of circulating folate and is an indicator of "average" folate status throughout the 24-hour interval (30). Urinary folate species of primary interest in the era of FA fortification (and/or among those using FA-containing supplements) are 5-methyl-THF, the main form in circulation, and FA.

Urinary 5-methyl-THF

Consumption of the study folate dose yielded urinary 5-methyl-THF that differed among physiologic groups (physiologic group x time, $P < 0.001$) after controlling for ethnicity, *MTHFR* C677T genotype, choline intake, and supplement use prior to study enrollment. Among lactating and nonpregnant women, urinary 5-methyl-THF excretion increased (both, $P \leq 0.001$) over the course of the study (**Figure 1.2A**). However, among pregnant women, urinary 5-methyl-THF did not change ($P = 1.0$) over the course of the study (Figure 1.2A). At study-end, pregnant women were excreting less 5-methyl-THF than lactating ($P = 0.006$) and nonpregnant ($P < 0.001$) women, which did not differ ($P = 0.251$) from each other (Figure 1.2A).

Urinary folic acid

Consumption of the study folate dose increased ($P < 0.001$) urinary FA excretion among nonpregnant women over the course of the study; however, FA excretion among pregnant and lactating women did not change ($P = 0.162–0.976$) over

the course of the study (Figure 1.2B). At study-end, pregnant and lactating women, which did not differ ($P = 0.941$) from each other, excreted less (both $P < 0.001$) urinary FA than nonpregnant women (Figure 1.2B). Unmetabolized FA was present in 77%, 89%, and 100% of 24-hour urine collections among pregnant, lactating, and nonpregnant women, respectively, at study-end.

Urinary total folate

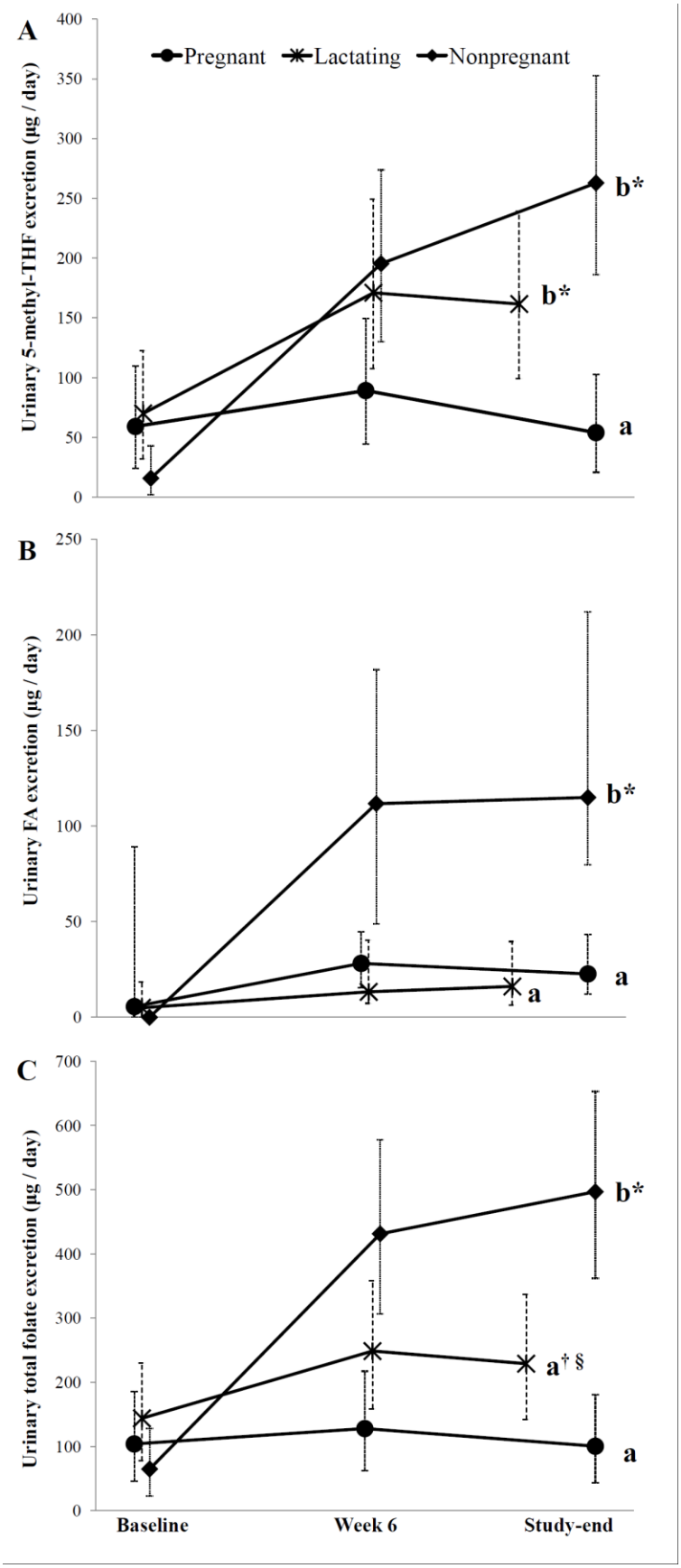
Similar to urinary 5-methyl-THF, consumption of the study folate dose yielded urinary total folate excretion that differed among physiologic groups (physiologic group x time, $P < 0.001$) after controlling for ethnicity, *MTHFR* C677T genotype, choline intake, and supplement use prior to study enrollment. Among nonpregnant women, urinary total folate increased ($P < 0.001$) over the course of the study, and among lactating women, urinary total folate tended to increase (P = 0.093) over the course of the study (Figure 1.2C). However, among pregnant women, urinary total folate did not change ($P = 1.0$) over the course of the study (Figure 1.2C).

At study-end, pregnant and lactating women excreted significantly less (both $P \leq 0.004$) total folate than nonpregnant women, and pregnant women tended to excrete less ($P = 0.075$) total folate than lactating women (Figure 1.2C).The study folate intake yielded substantial amounts of urinary folate at study-end, with mean urinary total folate excretion equivalent to approximately 9%, 20%, and 43% of daily total folate intake (1150 µg) for pregnant, lactating, and nonpregnant women, respectively.

Analyses with values adjusted with urinary creatinine excretion (31) to correct for possible incomplete 24-hour urine collection produced the same results for all urinary folate variables (data not shown).

Figure 1.2 legend

Urinary excretion of 5-methyl-THF (A); FA (B); and total folate (C) among third-trimester pregnant women (n = 26), lactating women 5–15 weeks postpartum (n = 28), and nonpregnant women of childbearing age (n = 21) consuming a FA-containing prenatal supplement plus 400 µg natural food folate for 10–12 weeks. Statistical analyses for 5-methyl-THF and total folate were performed with linear mixed models. Statistical analyses for FA were performed with Kruskal-Wallis and Mann-Whitney tests. All participants were included in the analyses. Plotted data for 5-methyl-THF and total folate excretion are back transformed predicted means and 95% confidence intervals derived from linear mixed models; medians with 95% confidence intervals were plotted for FA excretion. Study-end was week 10 for lactating participants, and week 12 for pregnant and nonpregnant participants. The interaction of physiologic state and time was significant ($P < 0.001$) for urinary 5-methyl-THF and total folate excretion; distribution of the urinary FA data did not allow for this interaction to be tested. Dissimilar letters indicate differences ($P < 0.01$) between physiologic groups at study-end. [†]indicates pregnant group and lactating group tended to be different ($P < 0.1$) at study-end. *indicates urinary folate concentrations increased ($P < 0.01$) from baseline to study-end within designated physiologic groups. § indicates folate excretion tended to increase ($P < 0.1$) from baseline to study-end within lactating group. To convert ng/mL to nmol/L folate, multiply ng/mL by 2.266. Abbreviations: 5-methyl-THF, 5-methyltetrahydrofolate; FA, folic acid


A
Pregnant
Lactating
Nonpregnant
Urinary 5-methyl-THF excretion (µg / day)
400
350
300
250
200
150
100
50
0
b*
b*
a
B
Urinary FA excretion (µg / day)
250
200
150
100
50
0
b*
a
a
C
Urinary total folate excretion (µg / day)
700
600
500
400
300
200
100
0
b*
a† §
a
Baseline
Week 6
Study-end

RBC Folate

RBC folate is an indicator of long-term folate status and is less sensitive to acute changes in folate intake (2).

Consumption of the study folate dose yielded RBC folate concentrations that differed among physiologic groups (physiologic group x time, $P = 0.002$) after controlling for ethnicity, *MTHFR C677T* genotype, choline intake, and supplement use prior to study enrollment. Among pregnant women and nonpregnant women, RBC folate concentrations increased ($P = 0.019$ and 0.044, respectively) slightly (~10%) over the course of the study, while among lactating women, RBC folate did not change ($P = 0.107$) over the course of the study (**Figure** 1.**3**).

At study-end, lactating women had higher ($P \leq 0.002$) RBC folate than both pregnant and nonpregnant women, and pregnant women had higher ($P < 0.001$) RBC folate than nonpregnant women (Figure 1.3). RBC folate concentrations are related to the 120 day lifespan of erythrocytes and may take up to 40 weeks to achieve steady state (32). Thus it is likely study participants had not fully acclimated to the study folate dose by study-end, and differences by physiologic group are not necessarily reflective of differences in physiologic utilization and/or requirement.

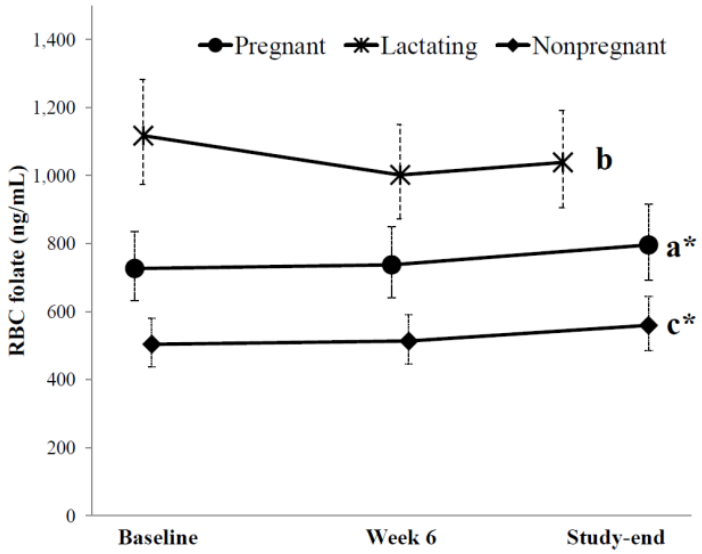


Figure 1.3 legend

RBC folate concentrations among third-trimester pregnant women (*n* = 26), lactating women 5–15 weeks postpartum (*n* = 28), and nonpregnant women of childbearing age (*n* = 21) consuming a FA-containing prenatal supplement plus 400 µg natural food folate for 10–12 weeks. Statistical analyses were performed with and reported from a linear mixed model. All participants were included in the analyses. Plotted data are back transformed predicted means and 95% confidence intervals derived from linear mixed models. Study-end was week 10 for lactating participants, and week 12 for pregnant and nonpregnant participants. The interaction of physiologic state and time was significant ($P = 0.002$). Dissimilar letters indicate differences ($P < 0.01$) between physiologic groups at study-end. *indicates RBC folate increased ($P < 0.05$) from baseline to study-end within designated physiologic groups. To convert ng/mL to nmol/L folate, multiply ng/mL by 2.266. Abbreviations: 5-methyl-THF, 5-methyltetrahydrofolate; FA, folic acid; RBC, red blood cell

Breastmilk folate

Breastmilk folate represents a route of folate loss among lactating women and is the primary source of folate for exclusively breastfed infants (2).

Consumption of the study folate dose did not alter breastmilk 5-methyl-THF ($P = 0.232$) or breastmilk total folate ($P = 0.244$) concentrations over the course of the study after controlling for ethnicity, *MTHFR* C677T genotype, choline intake, and supplement use prior to study enrollment (**Figure 1.4A**; **Table 1.2**). However, consumption of the study folate dose resulted in greater (time, $P < 0.001$) breastmilk FA concentrations over the course of the study (Figure 1.4A; Table 2). Moreover, supplement use prior to study enrollment interacted ($P < 0.001$) with study week (time) to affect breastmilk FA concentrations.

The relationship among supplement use prior to study enrollment, time and breastmilk FA concentrations was further explored because prior research has not shown a relationship between maternal folate (or FA) intake and breastmilk folate concentrations (33,34). Interestingly, breastmilk FA concentrations increased ($P = 0.001$) from baseline to study-end only among women not taking a supplement prior to study enrollment; there was no change ($P = 1.0$) in breastmilk FA concentrations among women taking a FA-containing supplement at baseline (Figure 1.4B; Table 1.2). At baseline, breastmilk FA concentrations were lower ($P < 0.001$) among women not consuming a supplement prior to study enrollment; however, at week 6 ($P = 0.324$) and study-end ($P = 0.796$), there were no differences in breastmilk FA concentrations based on supplement use prior to study enrollment (Figure 1.4B; Table 1.2). In addition, the ratio of breastmilk FA : breastmilk total folate was significantly

lower ($P < 0.001$) among lactating women not using a supplement prior to study enrollment at baseline; however, at week 6 ($P = 0.242$) and at study-end ($P = 0.174$) there were no differences in the breastmilk FA : breastmilk total folate ratio based on supplement use prior to study enrollment (Table 1.2). The ratio of breastmilk 5-methyl-THF : breastmilk total folate did not change ($P = 0.889$) over the course of the study.

TABLE 1.2 Breastmilk folate concentrations for lactating women 5–15 weeks postpartum (n = 28) at baseline and following the consumption of a FA-containing prenatal supplement plus 400 µg naturally occurring food folate for 10 weeks[1 2]

	Baseline	Study-end	*P*-value
5-methyl-THF (ng/mL)	14.0 (10.8 – 17.6)	16.3 (12.8 – 20.2)	0.232
Total folate (ng/mL)	56.2 (48.8 – 64.2)	61.8 (54.1 – 70.0)	0.244
FA (ng/mL)			
All participants	16.2 (11.8 – 21.3)	24.1 (18.7 – 30.3)	< 0.001
No supplement prior, n = 7	8.4 (3.5 – 15.4)[a]	21.4 (12.9 – 31.9)	< 0.001
Supplement prior, n = 21	26.6 (20.9 – 33.1)[b]	27.1 (21.3 – 33.6)	0.984
FA : total folate			
All participants	0.32 (0.26 – 0.39)	0.40 (0.34 – 0.46)	0.041
No supplement prior	0.18 (0.07 – 0.30)[a]	0.36 (0.25 – 0.46)	0.007
Supplement prior	0.46 (0.40 – 0.52)[b]	0.44 (0.38 – 0.50)	0.814

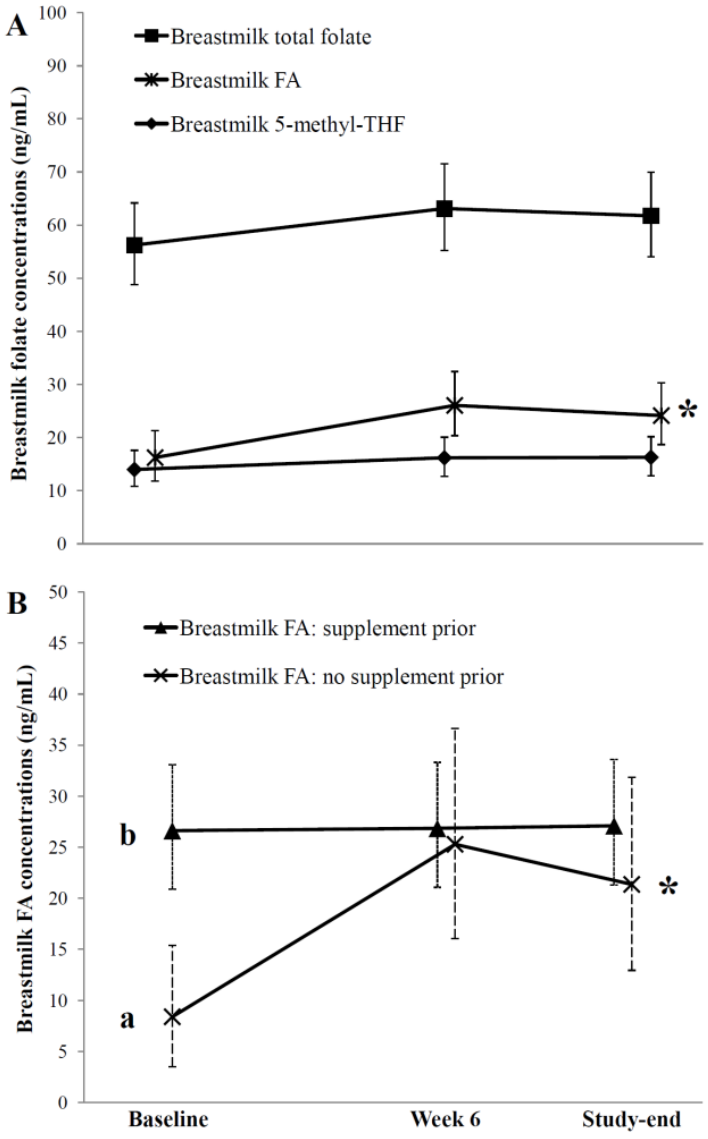
[1] Data were analyzed with linear mixed models; back transformed predicted means (95% confidence interval) are presented; *P* - value refers to baseline vs. study-end Bonferroni corrected pairwise comparison derived from linear mixed model; dissimilar superscripts within a column indicate baseline differences (Bonferroni corrected pairwise comparison at $P < 0.001$) between groups derived from linear mixed model.

[2] To convert ng/mL to nmol/L folate, multiply ng/mL by 2.266

Abbreviations used: 5-methyl-THF, 5-methyltetrahydrofolate; FA, folic acid

Figure 1.4 legend

Breastmilk 5-methyl-THF, total folate, and FA concentrations among lactating women (n = 28) consuming a FA-containing prenatal supplement plus 400 µg natural food folate for 10 weeks (A). Breastmilk FA concentrations comparing study participants that were consuming a FA-containing supplement prior to study enrollment (n = 21) to study participants that were not consuming a FA-containing supplement prior to study enrollment (n = 7) (B). Statistical analyses were performed with linear mixed models. All participants were included in the analyses. Plotted data are back transformed predicted means and 95% confidence intervals derived from linear mixed models. The interaction of breastmilk folic acid and supplement use prior to study enrollment was significant ($P < 0.001$). Dissimilar letters indicate differences ($P < 0.001$) between groups at designated time point.*indicates designated breastmilk folate increased ($P < 0.01$) from baseline to study-end. To convert ng/mL to nmol/L folate, multiply ng/mL by 2.266. Abbreviations: 5-methyl-THF, 5-methyltetrahydrofolate; FA, folic acid.


A
Breastmilk total folate
Breastmilk FA
Breastmilk 5-methyl-THF
Breastmilk folate concentrations (ng/mL)
100
90
80
70
60
50
40
30
20
10
0
*
B
Breastmilk FA: supplement prior
Breastmilk FA: no supplement prior
Breastmilk FA concentrations (ng/mL)
50
45
40
35
30
25
20
15
10
5
0
b
a
*
Baseline
Week 6
Study-end

DISCUSSION

This is the first controlled feeding study to comprehensively assess and compare folate status response to a known dose (FA-containing prenatal supplement plus 400 µg natural food folate) among third-trimester pregnant women, lactating women 5–15 weeks postpartum, and nonpregnant women of childbearing age. Three main findings emerged: (i) the study dose yielded supranutritional folate status in all women regardless of physiologic state; (ii) folate utilization is greater during the third-trimester of pregnancy than during lactation 5–15 weeks postpartum; and (iii) breastmilk folate species are responsive to maternal folate intake.

Consumption of the study folate dose yielded supranutritional folate status

Supranutritional folate status is indicated by folate status marker concentrations that (i) far surpass adequacy cut-offs and (ii) suggest metabolic capacity has been exceeded. Such status is achieved with folate intakes that are above what is reachable through consumption of natural food folate. Several biomarkers examined in the present study demonstrate that consumption of 400 µg natural food folate plus a FA-containing prenatal supplement yielded supranutritional status. First, study-end serum folate concentration estimates far exceeded 20 ng/mL, which is considered a marker of "high" folate status because it corresponds to the 95th percentile of population-wide folate status pre-FA fortification (5). Second, at study-end, 84% of women had detectable unmetabolized FA in fasted serum, and 100, 77, and 89% of nonpregnant, pregnant, and lactating women, respectively, excreted unmetabolized FA in their urine. Third, total urinary folate excretion represented ~9–43% of total folate intake, i.e. ~100–500 µg/day.

The impact of supranutritional folate status on health is unclear; however, most concerns regard masking of vitamin B12 deficiency, folate/ B12 imbalance, putative roles in cancer promotion, and exposure to unmetabolized FA (28). Many over-the-counter prenatal supplements contain 800–1000 μg FA (1360–1700 DFEs) (9), an amount that exceeds all recommended intakes for women of childbearing age and approaches/meets the 1000 μg tolerable upper level of intake (2). Given underlying folate sufficiency (5), current folate/FA intakes (35), and evidence of supranutritional folate status in the present study, reduction of prenatal supplement FA doses seems prudent.

Although conducted under controlled conditions, the results of this study are broadly generalizable in FA-fortified populations because study participants consumed an over-the-counter prenatal supplement and natural folate from a mixed diet. However, it should be noted that the supranutritional folate status achieved with this study's folate dose should not be extrapolated to less folate-replete populations.

Response by physiologic state

In the present study, intact 24-hour urinary folate excretion, which represents "average" folate status across the collection period, proved the most informative folate status marker (vs. fasted serum and RBC folate concentrations) in comparing folate utilization and requirement by physiologic group. Third-trimester pregnant women demonstrated the greatest folate utilization by excreting less urinary 5-methyl-THF and less total folate than lactating and nonpregnant women at study-end. Meanwhile, lactating women demonstrated greater folate utilization than nonpregnant women by excreting less urinary total folate and FA at study-end. The dose in the present study

far exceeded the current RDAs for pregnant and lactating women, thus in order to further refine RDA recommended intakes, dose response studies with intake levels approximating current RDAs and/or EARs are necessary.

Breastmilk folate

Results of the present study confirm other findings (33,34) that breastmilk total folate concentrations are maintained at constant levels regardless of maternal folate intake, i.e., breastmilk total folate concentrations did not vary based on supplement use prior to study enrollment nor were changes observed over the course of the study. However, results of the present study indicate, for the first time, that the distribution of folate species in breastmilk is related to maternal folate intake.

Previously published studies have reported 5-methyl-THF and FA make up 20–60% (36,37) and 8–40% (34,37) of breastmilk total folate, respectively, indicating a broad distribution of breastmilk folate species. A significant proportion of breastmilk folate is bound to the protein folate receptor α (FRα) (36,38). The amount of FRα in breastmilk is positively correlated ($r = 0.71$, $P < 0.001$) (38) with breastmilk total folate concentrations and FRα may play a role in regulating breastmilk folate concentrations (34,38). Among folate forms, FRα has the highest affinity for FA (39). In the present study, the study FA dose increased breastmilk FA, without altering breastmilk total folate concentrations, among lactating women who did not consume a FA-supplement prior to study-enrollment. The maintenance of breastmilk total folate concentrations in the midst of rising breastmilk FA concentrations implies that FA is displacing reduced folate forms from FRα in breastmilk. The high percentage of breastmilk FA (~40%) arising from the study FA dose may be of concern as

bioavailability studies with milk suggest that absorption of FA is less than 5-methyl-THF when bound to FRα (40).

Conclusions

Supranutritional folate status resulted from the consumption of a FA-containing prenatal supplement plus 400 µg natural food folate. At study-end, serum folate concentrations did not vary by physiologic group with the increased folate requirements of pregnant and lactating women only apparent via differences in urinary folate excretion. Breastmilk total folate concentrations did not change in response to the study folate dose; however, the proportion of FA in breastmilk increased. Given unresolved concerns about exposure to excess FA (for women of childbearing age as well as breastfeeding infants) and the widespread folate adequacy of our post-FA fortification population, it appears prudent to reduce the amount of FA in prenatal supplements to levels more in line with DRI recommended intakes.

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CHAPTER 2

Choline intake and pregnancy influence phosphatidylcholine docosahexaenoic acid enrichment among third trimester pregnant and nonpregnant women

ABSTRACT

Background: Phosphatidylcholine (PC) produced via the S-adenosylmethionine (SAM) dependant phosphatidylethanolamine (PE) *N*-methyltransferase (PEMT) pathway is enriched with docosahexaenoic acid (DHA). DHA plays a critical role in fetal development and is linked to cardiovascular and mental health endpoints in adults. It is unknown whether choline, which can serve as a source of SAM methyl donors, influences PC-DHA enrichment among pregnant and nonpregnant women.

Objective: This study tested whether choline intake impacted parameters of choline related lipid metabolism, i.e. erythrocyte and plasma PC-DHA and PC:PE ratio, among third-trimester pregnant and nonpregnant women.

Design: Pregnant (n =26) and nonpregnant (n =21) women consumed 430 or 930 mg choline/d and a daily DHA supplement under controlled intake conditions for 12 wks. Blood was collected at baseline, study-midpoint, and study-end.

Results: Pregnant women had greater (P= 0.01–0.001) PC-DHA than nonpregnant women at baseline. Erythrocyte and plasma PC-DHA increased ($P < 0.001$) among pregnant and nonpregnant women regardless of choline intake. However, among nonpregnant women consumption of 930 mg choline/d led to greater (P= 0.011) erythrocyte PC-DHA and a more rapid increase ($P< 0.001$) of plasma PC-DHA.

Lower (P= 0.001–0.015) erythrocyte and plasma PC:PE among pregnant women throughout the study was not modified by choline intake.

Conclusions: A higher choline intake may facilitate flux through the PEMT pathway resulting in greater PC-DHA concentrations and increased delivery of DHA to peripheral tissues among nonpregnant women. The increased production of PC-DHA during pregnancy is indicative of elevated PEMT activity and a higher demand for methyl donors. This trial was registered at clinicaltrials.gov as NCT01127022.

INTRODUCTION

The ubiquitous phospholipid phosphatidylcholine (PC) consists of 2 fatty acid constituents linked to a glycerophosphocholine backbone. The fatty acid composition of the PC molecule is a function of the pathway by which it is synthesized. PC produced via the phosphatidylethanolamine *N*-methyltransferase (PEMT) pathway is enriched in docosahexaenoic acid (DHA) (1) . In contrast, PC generated by the CDP-choline pathway is enriched in linoleic acid and oleic acid (1).

Adequate DHA nutrition is essential for health throughout the lifespan. The fetus requires DHA for proper development of the brain and immune systems (2,3). In adults, DHA nutrition is linked to inflammation, brain function, reproductive health, and cardiovascular disease (4). Following incorporation into VLDL, PC-DHA produced via PEMT is available for the fetus in pregnant women and transported to peripheral tissues in both pregnant and nonpregnant women. Thus, PEMT plays an essential role in mobilization and export of DHA from liver (5).

PEMT substrate concentrations including methyl donors (e.g. folate and the choline metabolite betaine) and supplementary DHA may influence PEMT activity and impact PC fatty acid composition in plasma and erythrocytes (6). However, the impact of a higher choline intake on PC-DHA enrichment in plasma and erythrocytes among pregnant and nonpregnant women is unknown. Erythrocyte PC-DHA enrichment is of particular interest as it is a more stable marker of status and is correlated with that of hepatic PC (7). In addition, erythrocyte DHA accretion correlates with brain, retina, and adipose DHA accretion (8).

The ratio of PC to PE in cellular membranes is tightly regulated (9,10). Changes in the PC:PE ratio can impair membrane functionality and a reduced membrane PC has been found during choline deficiency and in patients with non-alcoholic fatty liver disease (NAFLD), a condition associated with deranged choline metabolism (11,12). It is unknown whether choline intake impacts the PC:PE ratio in healthy pregnant and nonpregnant women, or whether the increased requirement for choline during pregnancy alters the PC:PE ratio in erythrocytes and/or plasma.

The primary aim of this study was to test whether choline intake under controlled conditions impacted erythrocyte and plasma PC fatty acid constituents and the PC:PE ratio among third-trimester pregnant and nonpregnant women. Because PEMT activity is upregulated by estrogen (13), which rises during pregnancy, a secondary aim was to investigate the impact of pregnancy on erythrocyte and plasma PC fatty acid constituents and PC:PE.

STUDY PARTICIPANTS AND METHODS

Study Participants

Nonpregnant and third trimester pregnant (27 weeks gestation) women aged ≥ 21 y were recruited from the Ithaca, NY area from January 2009–October 2010 as described by Yan et al. (14). During the screening phase, interested individuals provided a blood sample for blood chemistry profiling and complete blood count analyses, and completed a health history and demographics questionnaire (see Appendix B for questionnaires). Important inclusion criteria were: (i) general healthiness as determined by the questionnaire, blood chemistry profile, and complete blood count; (ii) no drug or alcohol use; (iii) normal kidney and liver function; and (iv) willingness to comply with study protocol, including agreement to eat ≥ 5 meals at the on-site location and not consume food or beverages outside what was provided by the study. Additional inclusion criteria for pregnant women were singleton pregnancy and no pregnancy associated complications, e.g., preeclampsia, gestational diabetes. Exclusion criteria included: inability to comply with study protocols and use of prescription medications known to affect liver function. Eligible pregnant women were admitted to the study on a rolling basis at 26–29 weeks gestation and eligible nonpregnant women were added as scheduling and space constraints allowed until the desired number of participants completed the study (14).

The study protocol was reviewed and approved by the Institutional Review Board for Human Study Participant Use at Cornell University and at the Cayuga Medical Center (the hospital where pregnant participants delivered their babies; Ithaca, NY). Study participants were compensated for participation and informed

consent was obtained from all participants prior to their entry into the study (see Appendix B for informed consent documents).

Study Design, Diet, and Supplements

Design

This study was part of a controlled feeding study conducted in nonpregnant and pregnant women randomized to consume 480 or 930 mg choline/d for 12 weeks (14) through a combination of dietary choline (~380 mg/day) and supplemental choline chloride (100 or 550 mg/day). Throughout the controlled feeding period all participants also consumed a daily 200 mg DHA supplement in order to achieve the recommended intake of this nutrient for pregnant women (15). Blood was collected and processed as previously described (14) at baseline and study weeks 6, 10, and 12.

Diet

The study diet provided ~380 mg choline/d (14). Lipid soluble forms of choline including PC, sphingomyelin, and lysophosphatidylcholine, contributed 236 mg choline/day, and water-soluble forms including free choline, phosphocholine, and glycerophosphocholine contributed 142 mg choline/d (14) (see Appendix B for study diet). In addition, the diet provided 100 mg betaine/day. The study diet supplied ~2000 kcal/d which could be modified to meet caloric requirements by the addition or subtraction of non-nutritive food items (14). Food was prepared in the Francis A. Johnston and Charlotte M. Young Human Metabolic Research Unit (HMRU) at Cornell University. Study participants consumed ≥ 5 meals/ week under the supervision of study personnel at the HMRU; all other food and beverages were provided as take-aways.

Supplements

Choline supplements were prepared by study personnel as described by Yan et al. (14). Briefly, pharmaceutical grade choline chloride (Balchem) was dissolved in autoclaved drinking water and amounts containing 100 mg and 550 mg choline were dispensed into sterile 50 mL conical tubes containing cranberry-grape juice and stored at -20^{o}C in HMRU freezers; 1-2 days prior to consumption, supplements were thawed at 4^{o}C

In addition, study participants consumed a daily 200 mg DHA supplement (Neuromins, Nature's Way Products, Springville, Utah), a daily over-the-counter prenatal multivitamin supplement (Pregnancy Plus®, Fairhaven Health, LLC, Bellingham, WA), and a thrice weekly potassium/magnesium supplement (General Nutrition Corp., Pittsburgh, PA) in order to achieve recommended nutrient intake levels not met with the study diet (15,16). When eating on-site, participants consumed supplements under the supervision of study personnel. Otherwise, supplements were provided in baggies along with take-away meals and participants were instructed to consume the supplements with a meal of their choice.

Compliance

The study protocol was well-tolerated with 92% of enrolled participants completing the study (21 of 22 nonpregnant and 26 of 29 pregnant). Reasons for stopping the study included nausea, early delivery, personal challenges, and food dislikes (14).

Study participants completed daily checklists indicating they received and consumed all menu items and supplements. For meals consumed off-site, participants

were asked to return all empty conical tubes, baggies, and take-away food containers to study personnel during their next visit to the HMRU. In addition, study personnel had daily contact with participants throughout the study to maintain positive rapport and enhance compliance.

Sample Collection and Processing

Fasting venous blood was drawn at baseline and study weeks 6, 10, and 12 in the HMRU ward by a trained phlebotomist. Blood samples were collected in EDTA and serum separator tubes, processed within 2 hours, and stored in cryostat tubes at -80 ^{o}C until analysis as previously described (14).

Analytic Measurements

Erythrocyte and plasma PC fatty acid constituents

Erythrocyte and plasma PC fatty acids constituents were quantified via gas chromatography-mass spectrometry (15). Fatty acid constituent data is presented as % total PC fatty acids.

Erythrocyte and plasma phospholipids

PC and PE were quantified from washed, packed red blood cells and plasma with a high performance liquid chromatography-evaporative light scattering detection method (17).

Genotyping

Select genetic variants impacting choline metabolism (methylenetetrahydrofolate reductase (*MTHFR*) C677T (18); methylenetetrahydrofolate dehydrogenase (*MTHFD1*) G11958A (19); and betaine-homocysteine *S*-methyltransferase (*BHMT*) G724A (20) were determined by

sequencing the double-stranded DNA templates with an Applied Biosystems Automated 3730 DNA analyzer.

Statistical Methods

The impact of choline intake and physiologic state on PC-DHA (22:6*n*-3) and PC-arachidonic acid (ARA, 20:4*n*-6) where examined separately. Additional dependant variables included: the sum of PC- eicosapentaenoic acid (EPA, 20:5*n*-3), PC-omega-3 docosapentaenoic acid (DPAn3, 22:5*n*-3), PC-omega-6 docosapentaenoic acid (DPAn6, 22:5*n*-6), and PC-docosatetraenoic acid (DTA, 22:4*n*-6); and an index of the main unsaturated species generated through the CDP-choline pathway, PC-linoleic acid (18:2*n*-6) and PC-oleic acid (18:1*n*-9).

Mann-Whitney U-tests were used to test baseline differences in participant characteristics and dependent variable values between physiologic groups and baseline differences by choline intake level within physiologic groups.

Linear mixed models (LMMs) were used to determine the impact of choline intake on dependant variables after grouping by physiologic state. In this set of LMMs, time (study week), choline intake (480 or 930 mg/day), and their interactions were entered as fixed factors; subject identifier was entered as random factor; the baseline dependant variable value was entered as covariate; and the interaction between baseline value and time was included. Plasma EPA+DPAs+DTA, plasma PC:PE, and erythrocyte PC:PE underwent natural log transformation prior to analysis with LMMs; all other dependent variables did not require transformation to satisfy model assumptions.

A second set of LMMs were used to illustrate the impact of physiologic state, i.e. pregnancy, on dependant variables over the course of the study. Physiologic state (nonpregnant or pregnant), time (study week), and their interactions were entered as fixed factors; subject identifier was entered as random factor; and choline intake (480 or 930 mg/day) was controlled for.

Additional covariates/factors considered in initial models included: age, ethnicity, BMI, serum folate concentrations, and the *MTHFR* C677T, *MTHFD1* G11958A, and/or *BHMT* G724A genotypes. Non-significant interactions were progressively removed until final models were derived. Bonferroni corrections were made for multiple comparisons where applicable.

All statistics were performed with IBM SPSS software (version 19; SPSS Inc, Chicago, IL).

RESULTS

Participant characteristics and baseline values

Forty-seven women were included in the final analyses. Twenty-one nonpregnant and 23 pregnant women completed 12 weeks of the study and 3 pregnant women completed 10 weeks of the study. The study-end time point was used in all statistical analyses and reflects the last sample collection for each study participant. The ethnicity/race of the study participants as well as *MTHFR* C677T, *MTHFD1* G11958A, and *BHMT* G724A genotypes were balanced by physiologic state and choline intake group (**Table 2.1**).

In erythrocytes at baseline, pregnant women had greater ($P = 0.001$) PC-DHA enrichment and lower ($P = 0.001$) PC-ARA enrichment than nonpregnant women; enrichment of other erythrocyte PC-fatty acid parameters did not differ (P = 0.487–0.674) (Table 2.1). In plasma, pregnant women had greater PC-DHA ($P = 0.010$) and PC-linoleic+oleic ($P = 0.047$), and lower PC-ARA ($P < 0.001$) and PC-EPA+DPAs+DTA ($P = 0.028$) enrichment than nonpregnant women (Table 2.1). Pregnant women had lower erythrocyte ($P < 0.001$) and plasma ($P = 0.014$) PC:PE than nonpregnant women at baseline (Table 2.1). Plasma PC-EPA+DPAs+DTA was lower ($P = 0.014$) among pregnant women randomized to the 930 mg choline/d group; no other dependant variables varied by choline intake at baseline (Table 2.1).

TABLE 2.1 Participant characteristics and baseline lipid values for pregnant women ~27 wk gestation and nonpregnant women of reproductive age randomized to consume 480 or 930 mg choline/day[12]

	Pregnant			Nonpregnant		
	480 mg/d	930 mg/d	All	480 mg/d	930 mg/d	All
Number of subjects[2]	13	13	26	10	11	21
Ethnicity (Caucasian /African American / Hispanic / Asian / Other)[2]	9/0/2/1/1	7/1/2/3/0	16/1/4/4/1	8/1/1/0/0	6/1/1/1/2	14/2/2/1/2
MTHFR genotype (CC / CT / TT)[2]	5/8/0	6/5/2	11/13/2	3/4/3	6/5/0	9/9/3
MTHFD1 genotype (GG / GA / AA)[2]	6/6/1	4/5/4	10/11/5	1/7/2	2/8/1	3/15/3
BHMT genotype (GG / GA / AA / undeterminable)[2]	8/3/1/1	7/2/1/3	15/5/2/4	4/6/0/0	9/1/1/0	13/7/1/0
Erythrocyte PC fatty acids (% of total)[3]						
DHA	3.1 ± 0.5	2.8 ± 0.8	2.9 ± 0.6	2.4 ± 0.8	2.1 ± 0.4	2.3 ± 0.6[a]

ARA	7.2 ± 1.4	7.6 ± 1.7	7.4 ± 1.6	8.6 ± 1.2	9.1 ± 1.5	8.9 ± 1.4[a]
EPA + DPAs + DTA	1.8 ± 0.4	1.9 ± 0.3	1.9 ± 0.3	1.7 ± 0.4	1.9 ± 0.4	1.8 ± 0.4
Linoleic + Oleic	35.6 ± 2.2	35.5 ± 2.2	35.6 ± 2.1	35.3 ± 2.1	37.0 ± 2.6	36.2 ± 2.5
Plasma PC fatty acids (% of total)[3]						
DHA	4.4 ± 0.8	3.8 ± 1.0	4.1 ± 0.9	3.6 ± 1.2	2.9 ± 0.4	3.2 ± 0.9[b]
ARA	12.4 ± 2.3	12.1 ± 2.3	12.2 ± 2.3	15.7 ± 1.7	16.5 ± 2.6	16.1 ± 2.2[a]
EPA + DPAs + DTA	2.3 ± 0.3	2.0 ± 0.2*	2.2 ± 0.3	2.4 ± 0.6	2.5 ± 0.6	2.4 ± 0.5[c]
Linoleic + Oleic	33.2 ± 2.4	33.6 ± 2.7	33.4 ± 2.5	31.9 ± 3.2	32.2 ± 3.2	32.0 ± 3.1[c]
Erythrocyte PC:PE[4]	1.0 (0.9–1.1)	1.0 (0.9–1.0)	1.0 (0.9–1.0)	1.2 (1.0–1.5)	1.2 (1.0–1.3)	1.2 (1.1–1.3)[a]
Plasma PC:PE[4]	40.4 (30.1–51.0)	41.6 (30.3–53.3)	40.6 (34.1–46.5)	63.6 (17.6–86.0)	59.8 (20.2–93.6)	62.1 (48.8–75.3)[c]

[1] Data were analyzed with Mann-Whitney U-tests.

[abc] All pregnant compared with all nonpregnant: [a] $P \leq 0.001$, [b] $P \leq 0.01$, [c] $P \leq 0.05$.

* Indicates difference within physiologic group between choline intake levels, $P < 0.05$.

[2] counts, [3] means ± st dev presented, or [4] medians (95% confidence interval)

Impact of Choline Intake on Erythrocyte and Plasma PC-fatty acids and PC:PE ratio

Third-trimester pregnant women

Erythrocytes: After controlling for baseline values and covariates, choline intake did not impact ($P = 0.543$) erythrocyte PC-DHA in pregnant women. However, choline intake and time tended to interact ($P = 0.066$) to influence erythrocyte PC-ARA (**Figure 2.1**). Erythrocyte PC-ARA enrichment did not differ by choline intake at week 6 ($P = 0.942$) or study-end ($P = 0.162$); however, PC-ARA decreased ($P = 0.02$) from week 6 to study-end only among women consuming 930 mg choline/d (430 mg choline/d group, $P = 0.286$). Choline intake did not impact erythrocyte PC-EPA+DPAs+DTA ($P = 0.827$) or PC-linoleic+oleic ($P = 0.246$) among pregnant women.

Plasma: After controlling for baseline values and covariates, choline intake did not impact plasma PC-DHA ($P = 0.480$), PC-ARA ($P = 0.990$), PC-EPA+DPAs+DTA ($P = 0.681$), or PC-linoleic+oleic ($P = 0.846$) enrichment among pregnant women.

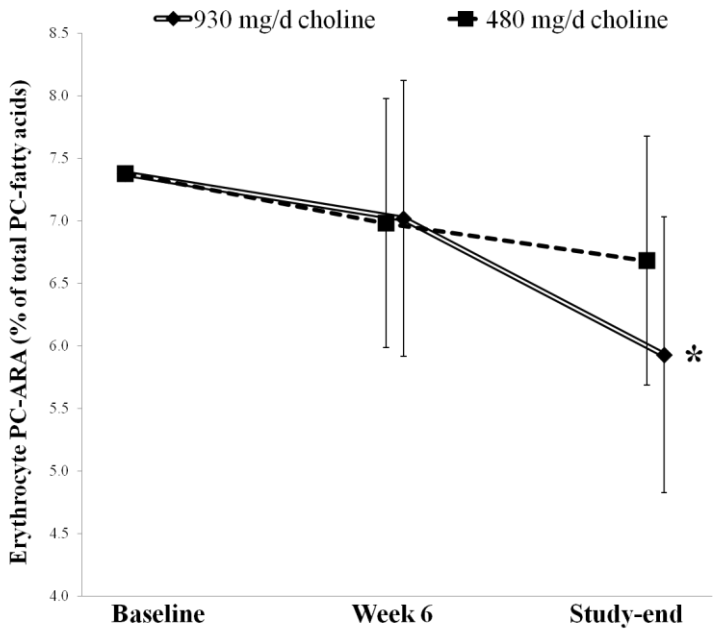


Figure 2.1 legend

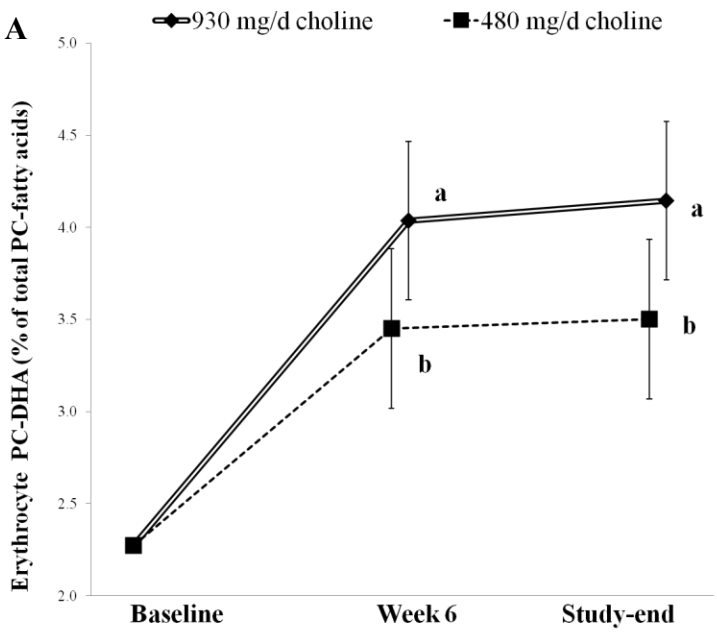
Erythrocyte PC-ARA among third-trimester pregnant consuming 480 (*n* = 13) or 930 (*n* = 13) mg choline/day under controlled conditions for 12 weeks. Statistical analyses were performed with linear mixed models that included baseline plasma PC-ARA as a covariate; plotted data are predicted means and 95% confidence intervals derived from linear mixed models. *indicates PC-ARA enrichment changed ($P < 0.01$) from week 6 to study-end. Abbreviations: ARA, arachidonic acid; PC, phosphatidylcholine

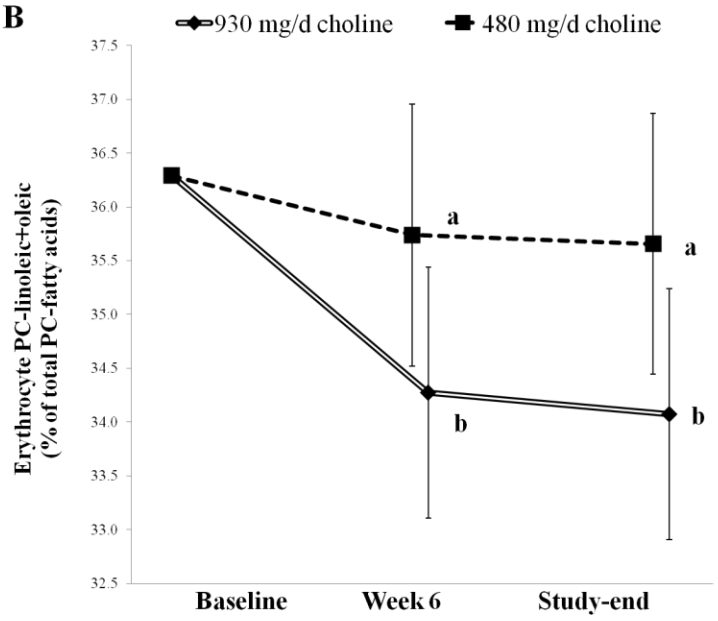
Nonpregnant women

Erythrocytes: After controlling for baseline values and covariates, nonpregnant women consuming 930 mg choline/d had greater (P = 0.011) erythrocyte PC-DHA than nonpregnant women consuming 480 mg/d through week 6 and study-end (**Figure 2.2A**). While choline intake did not impact (*P* = 0.131) erythrocyte PC-ARA enrichment, nonpregnant women consuming 930 mg choline/d tended (*P* = 0.096) to have greater PC-EPA+DPAs+DTA enrichment than women consuming 480 mg/d. Nonpregnant women consuming 930 mg choline/d had lower (*P* = 0.021) erythrocyte PC-linoleic+oleic than women consuming 480 mg/d through week 6 and study-end (Figure 2.2B).

Figure 2.2 legend

Erythrocyte PC-DHA (A) and erythrocyte PC-linoleic+oleic (B) among nonpregnant women consuming 480 (n = 10) or 930 (n = 11) mg choline/day under controlled conditions for 12 weeks. Statistical analyses were performed with linear mixed models that included baseline PC-DHA or PC-linoleic+oleic as a covariate; plotted data are predicted means and 95% confidence intervals derived from linear mixed models. Dissimilar letters indicate differences ($P < 0.05$) between groups. Abbreviations: DHA, docosahexaenoic acid; linoleic+oleic, linoleic acid + oleic acid; PC, phosphatidylcholine


A
930 mg/d choline
480 mg/d choline
Erythrocyte PC-DHA (% of total PC-fatty acids)
5.0
4.5
4.0
3.5
3.0
2.5
2.0
a
a
b
b
Baseline
Week 6
Study-end


B
930 mg/d choline
480 mg/d choline
Erythrocyte PC-linoleic+oleic (% of total PC-fatty acids)
37.5
37.0
36.5
36.0
35.5
35.0
34.5
34.0
33.5
33.0
32.5
a
a
b
b
Baseline
Week 6
Study-end

Plasma: After controlling for baseline values and covariates, choline intake level interacted with time ($P = 0.006$) to influence plasma PC-DHA enrichment (**Figure 2.3**). Nonpregnant women consuming 930 mg choline/d had greater ($P < 0.001$) PC-DHA than women consuming 480 mg/d at week 6; however, there was no difference ($P = 0.470$) in PC-DHA enrichment by choline intake level at study-end. Plasma PC-DHA decreased ($P = 0.035$) among nonpregnant women consuming 930 mg choline/d and increased ($P = 0.038$) among women consuming 480 mg choline/d from week 6 to study-end. Choline intake did not impact plasma PC-ARA ($P = 0.978$), PC-EPA+DPAs+DTA ($P = 0.263$), or PC-linoleic+oleic ($P = 0.606$) enrichment among nonpregnant women.

PC:PE ratio

After controlling for baseline values and covariates, choline intake did not impact erythrocyte ($P = 0.513–0.542$) or plasma ($P = 0.399–0.615$) PC:PE in pregnant or nonpregnant women.

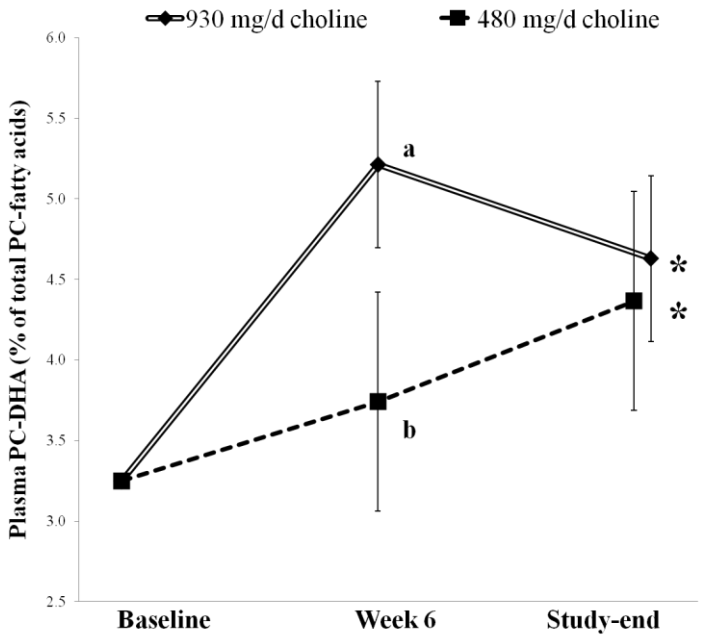


Figure 2.3 legend

Plasma PC-DHA among nonpregnant women consuming 480 (n = 10) or 930 (n = 11) mg/d choline under controlled conditions for 12 weeks. Statistical analyses were performed with linear mixed models that included baseline PC-DHA as a covariate; plotted data are predicted means and 95% confidence intervals derived from linear mixed models. Dissimilar letters indicate differences (P < 0.001) between groups. *indicates PC-DHA enrichment changed (P < 0.05) from week 6 to study-end. Abbreviations: DHA, docosahexaenoic acid; PC, phosphatidylcholine

Impact of Pregnancy on Erythrocyte and Plasma PC-fatty acids and PC:PE ratio

Erythrocytes: After controlling for covariates, physiologic state interacted with time ($P < 0.001$) to influence erythrocyte PC-DHA (**Figure 2.4**). At baseline pregnant women had greater ($P = 0.003$) erythrocyte PC-DHA enrichment than nonpregnant women; however, by week 6 and at study-end there was no difference ($P = 0.835$) by physiologic state. Erythrocyte PC-DHA enrichment increased ($P < 0.001$) from baseline to study-end in both pregnant and nonpregnant groups. Pregnant women had lower erythrocyte ($P = 0.005$) PC-ARA than nonpregnant throughout the study; erythrocyte PC-ARA decreased ($P = 0.031$) from baseline to study-end among all women. Erythrocyte PC-EPA+DPAs+DTA and PC-linoleic+oleic enrichment did not vary by physiologic group ($P = 0.190–0.538$) and decreased ($P = 0.031–0.033$) among all women over the course of the study. **Figure 2.5** summarizes erythrocyte PC-fatty acid distribution at baseline and study-end.

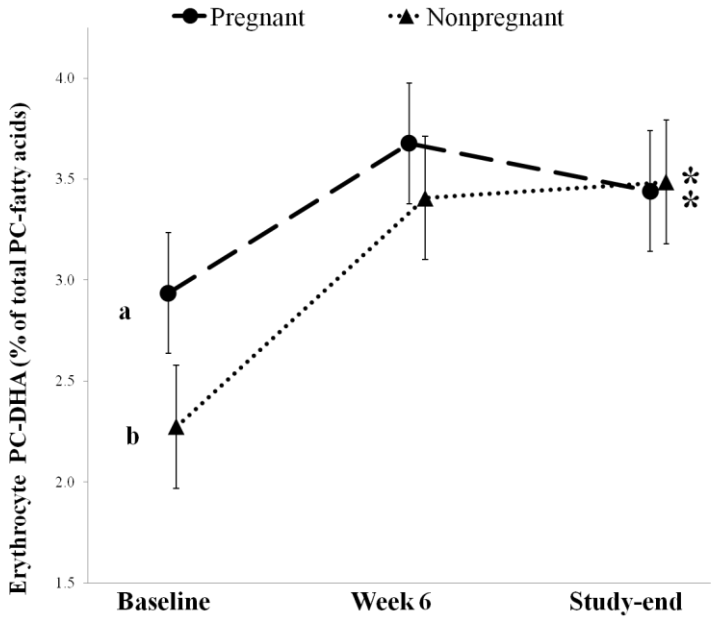


Figure 2.4 legend

Erythrocyte PC-DHA among third-trimester pregnant (n = 26) and nonpregnant women (n = 21) consuming a constant choline intake for 12 weeks. Statistical analyses were performed with linear mixed models; plotted data are predicted means and 95% confidence intervals derived from linear mixed models. Dissimilar letters indicate differences ($P < 0.01$) between pregnant and nonpregnant groups. *indicates PC-DHA enrichment changed ($P \leq 0.001$) from baseline to study-end. Abbreviations: DHA, docosahexaenoic acid; PC, phosphatidylcholine

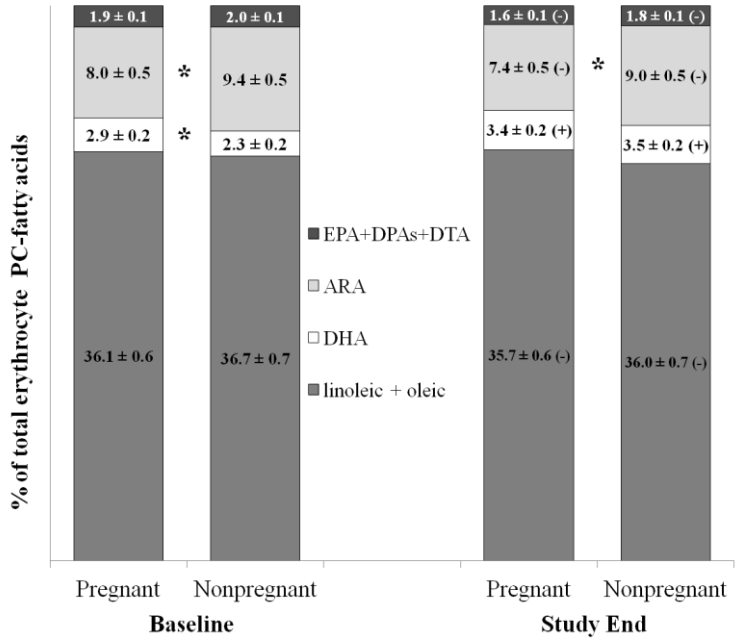


Figure 2.5 legend

Erythrocyte PC-fatty acids among third-trimester pregnant (n = 26) and nonpregnant women (n = 21) at baseline and after consuming a constant choline intake for 12 weeks. Statistical analyses were performed with linear mixed models; plotted data are predicted means and 95% confidence intervals derived from linear mixed models. * indicates difference ($P < 0.01$) between pregnant and nonpregnant groups. (-) and (+) at study-end indicate negative or positive change ($P < 0.05$) from baseline. Abbreviations: ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA+DPAs+DTA, eicosapentaenoic acid + omega-3 docosapentaenoic acid + omega-6 docosapentaenoic acid + docosatetraenoic acid; PC, phosphatidylcholine

Plasma: After controlling for covariates, physiologic state interacted with time ($P < 0.001$) to influence plasma PC-DHA (**Figure 2.6**). At baseline pregnant women tended to have greater ($P = 0.065$) plasma PC-DHA than nonpregnant women; however, by week 6 and at study-end there was no difference ($P = 0.163$) by physiologic state. Plasma PC-DHA enrichment increased ($P \leq 0.001$) from baseline to study-end in both pregnant and nonpregnant groups. Pregnant women had lower plasma PC-ARA ($P < 0.001$) and PC-EPA+DPAs+DTA ($P < 0.001$) than nonpregnant women throughout the study; among all women, plasma PC-ARA and PC-EPA+DPAs+DTA decreased ($P = 0.002$ and $P < 0.001$, respectively) from baseline to study-end. Physiologic state tended to interact with time ($P = 0.068$) to influence plasma PC-linoleic+oleic. At baseline pregnant women tended to have greater ($P = 0.079$) plasma PC-linoleic+oleic than nonpregnant women and this difference became significant ($P < 0.001$) by study-end. Plasma PC-linoleic+oleic did not change ($P = 0.633$) from baseline to study-end among pregnant women, but decreased ($P = 0.003$) among nonpregnant women during the same period. **Figure 2.7** summarizes plasma PC-fatty acid distribution at baseline and study-end**.**

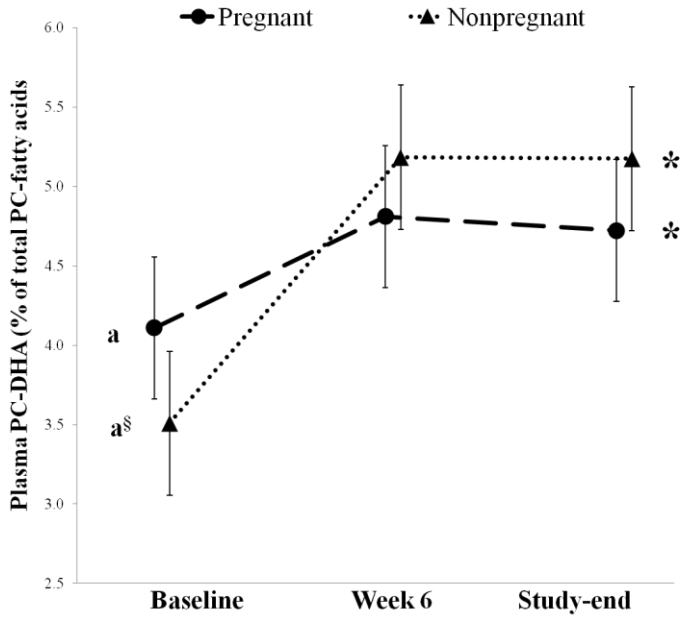


Figure 2.6 legend

Plasma PC-DHA among third-trimester pregnant (*n* = 26) and nonpregnant women (*n* = 21) consuming a constant choline intake for 12 weeks. Statistical analyses were performed with linear mixed models; plotted data are predicted means and 95% confidence intervals derived from linear mixed models. [§]indicates a borderline difference ($P < 0.1$) between pregnant and nonpregnant groups. *indicates PC-DHA enrichment changed ($P < 0.01$) from baseline to study-end. Abbreviations: DHA, docosahexaenoic acid; PC, phosphatidylcholine

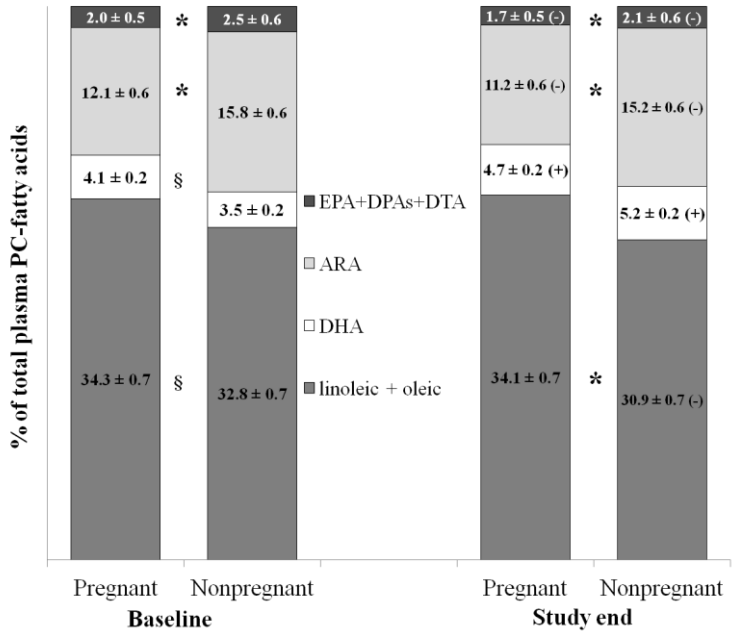


Figure 2.7 legend

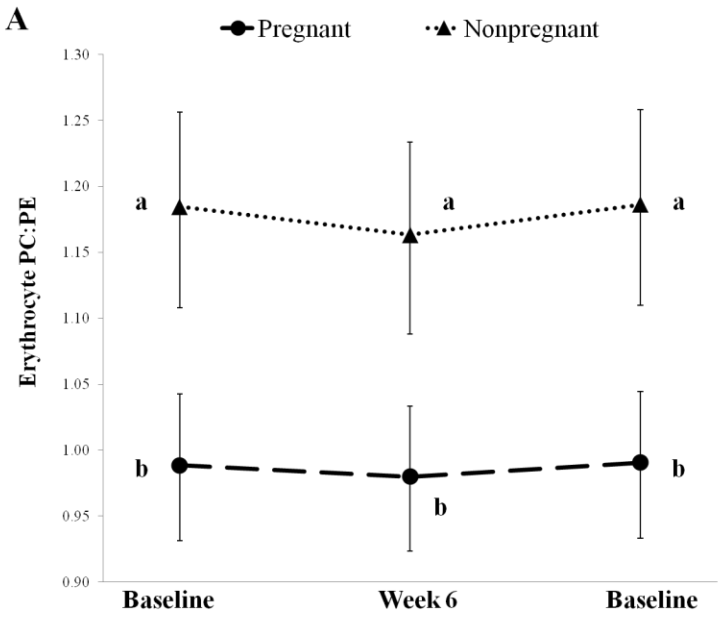
Plasma PC-fatty acids among third-trimester pregnant (*n* = 26) and nonpregnant women (*n* = 21) at baseline and after consuming a constant choline intake for 12 weeks. Statistical analyses were performed with linear mixed models; plotted data are predicted means and 95% confidence intervals derived from linear mixed models. *indicates difference ($P < 0.001$) between pregnant and nonpregnant groups. §indicates a borderline difference ($P < 0.1$) between pregnant and nonpregnant groups. (-) and (+) at study-end indicate negative or positive change ($P < 0.01$) from baseline. Abbreviations: ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA+DPAs+DTA, eicosapentaenoic acid + omega-3 docosapentaenoic acid + omega-6 docosapentaenoic acid + docosatetraenoic acid; linoleic + oleic, linoleic acid + oleic acid; PC, phosphatidylcholine

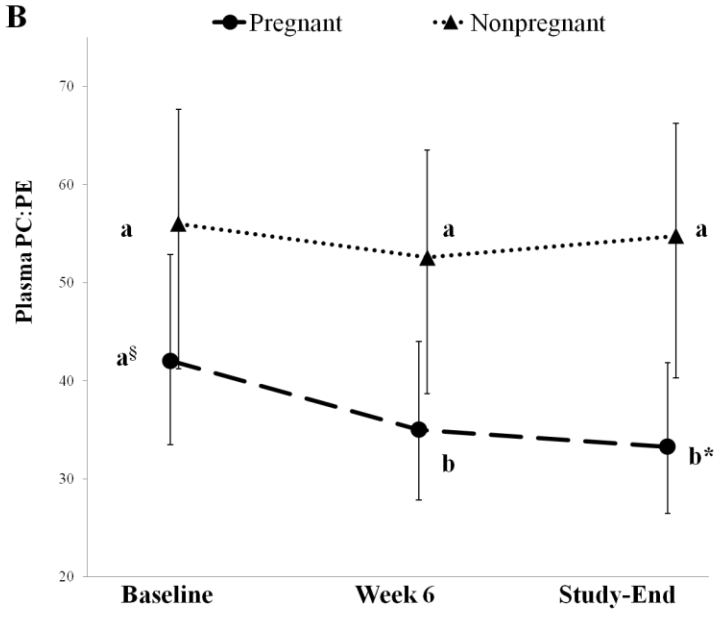
PC:PE ratio

Throughout the study, pregnant women had lower erythrocyte ($P < 0.001$) and plasma ($P = 0.015$) PC:PE than nonpregnant women. Erythrocyte PC:PE did not change ($P = 0.546$; **Figure 2.8A**) over the course of the study; however, in plasma, physiologic state interacted with time ($P = 0.039$; Figure 2.8B) to influence the PC:PE ratio response. Among pregnant women, plasma PC:PE decreased ($P < 0.001$) from baseline to study-end; however, among nonpregnant women this ratio did not change ($P = 1.0$). Among pregnant women, both plasma PC and PE increased ($P = 0.001$–0.007) from baseline to study-end; however, PC increased by ~11% while PE increased by 30%, thus the PC:PE ratio decreased among pregnant women. Among nonpregnant women, neither plasma PC ($P = 0.796$) nor plasma PE ($P = 0.705$) changed from baseline to study-end.

Figure 2.8 legend

Erythrocyte PC:PE (A) and plasma PC:PE (B) among third-trimester pregnant (*n* = 26) and nonpregnant women (*n* = 21) consuming a constant choline intake for 12 weeks. Statistical analyses were performed with linear mixed models; plotted data are back transformed predicted means and 95% confidence intervals derived from linear mixed models. Dissimilar letters indicate differences ($P < 0.05$) between pregnant and nonpregnant groups. [§]indicates a borderline difference ($P < 0.1$) between pregnant and nonpregnant groups. *indicates PC:PE ratio changed ($P < 0.001$) from baseline to study-end. Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine


A
Pregnant
Nonpregnant
Erythrocyte PC:PE
1.30
1.25
1.20
1.15
1.10
1.05
1.00
0.95
0.90
a
a
a
b
b
b
Baseline
Week 6
Baseline


B
Pregnant
Nonpregnant
Plasma PC:PE
70
60
50
40
30
20
a
a
a
a§
b
b*
Baseline
Week 6
Study-End

DISCUSSION

Due to its highly controlled nature, this study offered a unique opportunity to examine the interrelationships of choline and lipid metabolism in pregnant and nonpregnant women. This analysis compared parameters of choline related lipid metabolism among: (i) third trimester pregnant women consuming 480 or 930 mg choline/d; (ii) nonpregnant women consuming 480 or 930 mg choline/d; and (iii) third trimester pregnant and nonpregnant women. Three main findings emerged: (i) among nonpregnant women, the rise in PC-DHA was augmented by a higher choline intake; (ii) supplementary DHA may influence PEMT activity; and (iii) pregnancy was associated with changes in choline related lipid metabolism.

Choline intake influenced PC-DHA among nonpregnant women

Among nonpregnant women, a higher choline intake yielded greater erythrocyte PC-DHA throughout the study and a more rapid increase in plasma PC-DHA. Changes in erythrocyte PC-fatty acids among women consuming 930 mg choline/d suggest that as flux through the PEMT pathway increased (shown by greater PC-DHA, Figure 2A and greater ($P = 0.096$) PC-EPA+DPAs+DTA), flux through the CDP-choline pathway concurrently decreased (shown by lower PC-linoleic+oleic, Figure 2B). Thus, while the study treatment which included supplementary DHA increased erythrocyte and plasma PC-DHA among all nonpregnant women, these results indicate a higher choline intake and supplementary DHA acted synergistically to yield the greatest enrichment of erythrocyte PC-DHA.

A higher choline intake may facilitate increased flux through the PEMT by increasing the availability of methyl donors via its metabolite betaine. Indeed, studies

using isotopically labeled choline (d9-choline) have confirmed that methyl groups derived from the choline molecule are used in converting PE to PC via the PEMT pathway (21,22). In addition, a recent animal study found that female mice rely more heavily (vs. males) on choline derived methyl groups for the production of PC through SAM dependant PEMT (21). 5-methyltetrahydrofolate (5-methyl-THF) is the other carrier of labile methyl groups used to generate SAM. In the present study, folate status was "supranutritional" among nonpregnant women as evidenced by high serum concentrations and urinary excretion of ~40% of daily folate intake (23). Importantly, serum 5-methyl-THF did not differ ($P = 0.328$) by choline intake group. Thus, among nonpregnant women, consumption of 930 vs. 480 mg choline/d yielded greater erythrocyte PC-DHA and faster enrichment of plasma PC-DHA despite plentiful 5-methyl-THF, suggesting a unique relationship between increased availability of choline derived methyl groups and flux through PEMT.

DHA as a driver of PEMT

Consumption of the study DHA supplement increased erythrocyte and plasma PC-DHA among pregnant and nonpregnant women regardless of choline intake. The use of plasma PC-DHA as an indicator of hepatic PEMT activity has been suggested by da Costa et al. (24). Increases in plasma PC-DHA, ~15% and ~48% among pregnant and nonpregnant women, respectively, and erythrocyte PC-DHA, ~17% and ~53% among pregnant and nonpregnant women, respectively, suggests that supplementary DHA increases flux through the PEMT pathway. Nobili et al. (25) found supplementary DHA improved liver steatosis in children with NAFLD, which supports the hypothesis that increased DHA drives PEMT activity, i.e. as a substrate,

supplementary DHA may increase PEMT pathway activity, and in the case of Nobili et al. (25), improve dysfunctional VLDL formation associated with NAFLD.

Although improved and/or increased VLDL formation via increased PEMT activity among patients with NAFLD may reduce steatosis, it is not clear that such changes in hepatic lipid metabolism would benefit healthy individuals (26). In the present study, study-end plasma PC-DHA was not correlated (Pearson coefficient, 0.199; $P = 0.387$) with study-end LDL in nonpregnant women, suggesting increased PEMT pathway activity altered the make-up but not quantity of VLDL formed among these healthy women. Interestingly, among pregnant women at study-end, plasma PC-DHA was positively correlated (Pearson coefficient, 0.499; $P = 0.010$) with study-end LDL; however, this relationship is likely not caused by supplementary DHA increasing PEMT activity. Other mechanisms related to pregnancy induced hyperlipidemia, including increased adipose lipolysis, lead to the markedly increased VLDL production known to occur during the third-trimester of pregnancy (27).

Choline related lipid metabolism during pregnancy

Pregnancy impacted several parameters of choline related lipid metabolism including erythrocyte and plasma PC-fatty acid enrichment and PC:PE. As PC-DHA is primarily produced via the PEMT pathway, the higher PC-DHA in both erythrocytes and plasma at baseline among pregnant women is consistent with upregulated PEMT related to elevated estrogen. Pregnancy induced upregulation of PEMT may explain why choline intake did not impact erythrocyte or plasma PC-DHA enrichment among this group of pregnant women and why erythrocyte and plasma PC-DHA enrichment increased less (erythrocyte: 17% vs. 53% and plasma 15% vs. 48%) than enrichment

among nonpregnant women consuming the same DHA supplement. That is, if PEMT was already upregulated at baseline (i.e. week 27 gestation), it is possible that the capacity of choline derived methyl donors and/or additional DHA substrate to influence PC biosynthesis via PEMT was reduced. Nevertheless, elevated activity of the PEMT pathway, a significant consumer of SAM (28), underscores the increased demand for methyl groups during pregnancy. Additional studies are needed to examine whether a higher choline intake may enhance PEMT activity and PC-DHA enrichment at early stages in gestation.

Both erythrocyte and plasma PC:PE were lower among third-trimester pregnant women as compared with nonpregnant women. It is of note that neither these differences nor the decrease of plasma PC:PE observed among pregnant women were not modified by a higher choline intake. This indicates that the altered PC:PE ratio during pregnancy is not governed by choline intake/availability and may be due to conditions associated with this physiologic state such as insulin resistance (29), changes in PE biosynthesis (30), and/or reduce hepatic lipase activity (31). At this time it is unclear whether the reduced PC:PE ratio during pregnancy has functional consequences and it is unknown for how long the lower PC:PE ratio persists after parturition.

Conclusions

Increasing the level of circulating PC-DHA is often desirable among pregnant and nonpregnant women. The strong specificity for PC-DHA generation via the SAM dependant PEMT pathway increases the use of and demand for methyl groups, with perhaps an explicit requirement for methyl groups derived from choline. Thus, efforts

to better understand and/or improve DHA nutriture should fully consider factors influencing the PEMT pathway, including methyl donors such as choline.

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CHAPTER 3

Comparison of microbiological assay and LC-MS/MS for quantification of folates in serum, urine, and breastmilk

ABSTRACT

Background: The comparison of the microbiological assay (MA), a well established method for total folate quantification, and more recently developed liquid chromatography dual mass spectrometry (LC-MS/MS) techniques that quantify individual folate species is of methodologic interest.

Methods: Serum, urine, and breastmilk samples collected over the course of a controlled feeding study were subjected to MA total folate quantification. 5-methyltetrahydrofolate (5-methyl-THF) and folic acid (FA) were quantified via LC-MS/MS in the same samples. Bland Altman limits of agreement (LoA) calculations were performed to compare total folate measured via MA and the sum of 5-methyl-THF and FA measured via LC-MS/MS.

Results: The LoA mean differences (95% intervals) between MA total folate and the sum of 5-methyl-THF + FA measured in serum, urine, and breastmilk were -1.0 ng/mL (4.5, -6.6); 54.8 µg/day (260.0, -150.5 µg/day); and 20.2 ng/mL (44.6, -4.2 ng/mL), respectively, with MA as the reference value. MA and LC-MS/MS values were correlated; however, measurements from the two methods were significantly different for all three sample types. Differences between MA and LC-MS/MS methods in urine demonstrated a concentration dependant relationship.

Conclusions: In fasted serum, total folate measured via MA is comparable to the sum of 5-methyl-THF + FA measured via LC-MS/MS. Larger differences between MA and LC-MS/MS measurements in urine and breastmilk indicate the presence of additional folate species. Recommendations for improving the agreement of MA and LC-MS/MS methods in urine and breastmilk include monitoring of 5-methyl-THF-diglutamate in urine samples and extracting folate from > 4 mL breastmilk for improved sensitivity.

BACKGROUND

The microbiological assay (MA) has been described as the "gold standard" for quantification of total folate in biological samples, i.e., the sum of all biologically active folate forms; however, more recently developed liquid chromatography dual mass spectrometry (LC-MS/MS) methods are appealing because individual folate species, e.g., 5-methyltetrahydrofolate (5-methyl-THF) and folic acid (FA), can be measured. Therefore, it is of interest whether the sum of individual folate species measured via LC-MS/MS is equivalent to total folate measured via MA (1). Prior studies (2) have compared serum folate concentrations quantified via MA and LC-MS/MS; however, comparison of MA and LC-MS/MS folate quantification methods have not been published for urine or breastmilk samples.

The Bland-Altman Limits of Agreement Method (LoA) is an accepted means by which to evaluate the agreement of two methods measuring the same quantity (3). Using pairs of measurements (i.e., one value per method), LoA generates the mean difference between the two methods and an interval based on the standard deviation that contains 95% of pairs; the measurement pairs, mean difference, and 95% interval can also be represented graphically (3). In this study, 5-methyl-THF and FA were quantified in serum, urine, and breastmilk using LC-MS/MS methods, and total folate was quantified in the same samples using the MA. The Bland-Altman LoA method was used to compare the sum of 5-methyl-THF + FA to the total folate value, using MA total folate as the reference value.

MATERIALS AND METHODS

Samples

MA and LC-MS/MS measurements were performed with serum, urine, and breastmilk samples collected during a 10-12 week controlled feeding study conducted in women of childbearing age described in detail elsewhere (4,5).

Microbiological assay measurements

The MA (6) with *Lactobacillus casei* (ATCC 7469) as the test organism and 10 ng/mL FA (Sigma) as the standard calibrator was used to determine total folate in serum, urine, and breastmilk. Breastmilk samples were subjected to trienzyme digestion as described in West et al. (5) prior to measurement via MA. The coefficients of variance (CVs) were calculated using a positive quality control sample of each analyte. MA intra-assay CVs were < 10%. MA inter-assay CVs were as follows: serum, whole blood, and breastmilk, <10%; urine and dietary folate, <13%.

LC-MS/MS measurements

5-methyl-THF and FA were quantified using LC-MS/MS stable-isotope dilution methods as described in West et al. (5). Briefly, serum, urinary, and breastmilk extracts were prepared using solid phase extraction clean-up methods on a 12-port vacuum manifold (J.T. Baker, Inc.). Standard curves were prepared from 5-methyl-THF (Sigma) and FA (Sigma) stock solutions and internal standard solutions were prepared using ^{13}C-5-methyl-THF and ^{13}C-FA (Merck Eprova, Switzerland) as in Pfeiffer et al. (2). Serum and breastmilk 5-methyl-THF and FA were quantified with a TSQ Quantum mass spectrometer (Thermo, San Jose, CA) equipped with a refrigerated Accela autosampler (Thermo) and Accela pump with degasser (Thermo).

Urinary 5-methyl-THF and FA were quantified with a a LCQ Advantage Max mass spectrometer (Thermo) equipped with a refrigerated Surveyor autosampler (Thermo) and Surveyor pump with degasser (Thermo). Intra- and inter-assay CVs were <10% and <15%, respectively.

Statistical analysis

Bland-Altman LoA calculations and statistics were performed with Microsoft Excel 2007 and IBM SPSS software (version 19; SPSS Inc, Chicago, IL) as described in (3,7) correcting for multiple observations per individual (7).

RESULTS

Serum: Three different samples from 47 participants (141 pairs) were used to calculate the serum LoA . The mean difference (95% interval) between serum total folate measured via MA and serum 5-methyl-THF + FA measured via LC-MS/MS was -1.0 ng/mL (4.5, -6.6) (**Figure 3.1A**); although very close, this difference was significant ($P < 0.001$). The 141 pair Pearson correlation was 0.96 ($P < 0.001$).

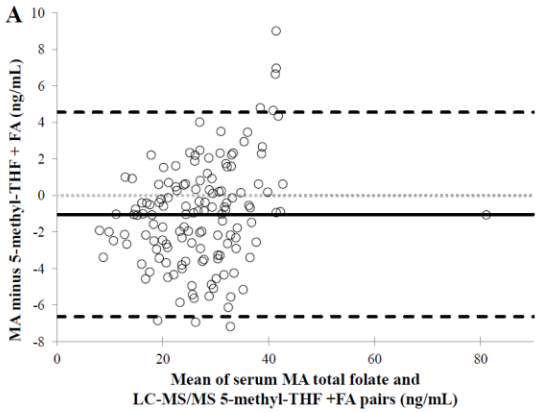
Urine: Three different samples from 75 participants (225 pairs) were used to calculate the urine LoA. The mean difference (95% interval) between urinary total folate measured via MA and urinary 5-methyl-THF + FA measured via LC-MS/MS was 54.8 µg/day (260.0, -150.5 µg/day) (Figure 3.1B); this difference was significant ($P < 0.001$). Due to the extreme range of urinary folate values and the appearance of a concentration dependant difference between the methods, an additional LoA graph using log-transformed data was generated (Figure 3.1C) (3). Figure 3.1C illustrates the concentration dependant relationship ($y = -0.23x + 0.69$ ng/mL, $r^2 = 0.32$) where measurements of greater urinary folate excretion had better agreement, i.e. the mean difference between the MA and LC-MS/MS methods was closer to 0 for samples with greater folate concentrations. MA and LC-MS/MS measurement pair averages (the mean MA and LC-MS/MS x-axis coordinates in LoA figures) were positively correlated with the proportion of FA to MA total folate (rho = 0.728, $P < 0.001$) and 5-methyl-THF to MA total folate (rho = 0.272, $P < 0.001$). In other words, as 5-methyl-THF and FA made up a greater proportion of urinary folate, the sum of the LC-MS/MS measurement matched the total folate measurement more closely (Figure 3.1C). The 225 pair Pearson correlation was 0.87 ($P < 0.001$).

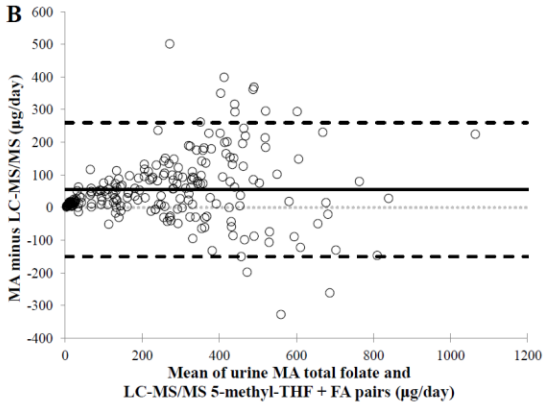
Breastmilk: Three different samples from 28 participants (84 pairs) were used to calculate the LoA. The mean difference (95% interval) between breastmilk total folate measured via MA and breastmilk 5-methyl-THF + FA folate measured via LC-MS/MS was 20.2 ng/mL (44.6, -4.2 ng/mL) (Figure 3.1D); this difference was significant ($P < 0.001$). The 84 pair Pearson correlation was 0.88 ($P < 0.001$).

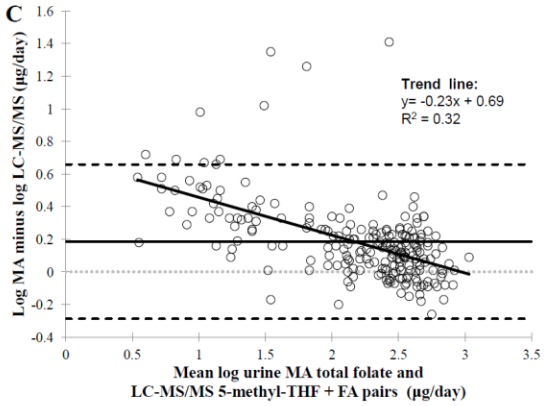
Figure 3.1legend

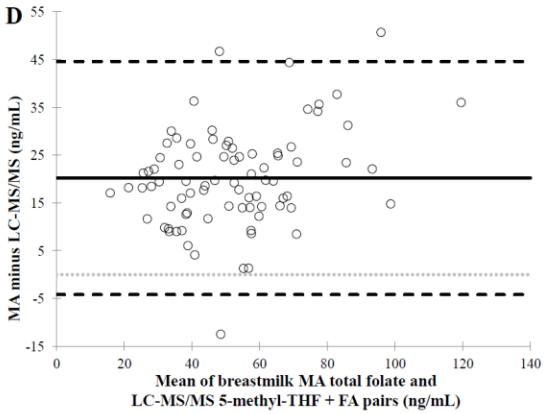
Bland-Altman Limit of Agreement (LoA) plots for serum (A); urinary (B); log-transformed urinary (C); and breastmilk (D) MA and LC-MS/MS measurements. LoA plots were generated with pairs (A = 141; B = 225; C = 225; D = 84) consisting of one MA total folate measurement and one LC-MS/MS 5-methyl-THF + FA measurement. Solid horizontal lines indicate mean difference of total folate measured via MA and 5-methyl-THF + FA measured via LC-MS/MS. Dashed horizontal lines indicate interval that contains 95% of points. The trend line in (C) indicates a concentration dependant relationship between the magnitude of urinary folate excretion and the agreement/difference of MA vs. LC-MS/MS method.

Abbreviations: 5-methyl-THF, 5-methyltetrahydrofolate; FA, folic acid; MA, microbiological assay; LoA, Bland-Altman Limit of Agreement plot; LC-MS/MS, liquid chromatography-dual mass spectrometry


A
MA minus 5-methyl-THF + FA (ng/mL)
Mean of serum MA total folate and
LC-MS/MS 5-methyl-THF +FA pairs (ng/mL)


B
MA minus LC-MS/MS (µg/day)
Mean of urine MA total folate and
LC-MS/MS 5-methyl-THF + FA pairs (µg/day)


C
Log MA minus log LC-MS/MS (µg/day)
Trend line:
y= -0.23x + 0.69
R² = 0.32
Mean log urine MA total folate and
LC-MS/MS 5-methyl-THF + FA pairs (µg/day)


D
MA minus LC-MS/MS (ng/mL)
Mean of breastmilk MA total folate and
LC-MS/MS 5-methyl-THF + FA pairs (ng/mL)

DISCUSSION

In serum samples, total folate measured via MA and 5-methyl-THF + FA measured via LC-MS/MS match well, although the sum of folate species measured via LC-MS/MS was significantly greater than MA total folate. In contrast, Pfeiffer et al. found that serum total folate measured via MA was significantly greater than the sum of serum 5-methyl-THF, FA, and 5-formyltetrahydrofolate (5-formyl-THF) measured via LC-MS/MS; however, the difference was dependent upon the folate species used to calibrate the MA (2). Pfeiffer et al. found that 2.5–4.4% of non-fasted serum folate was present as 5-formyl-THF (2); the amount of 5-formyl-THF in fasted serum in the present study is not known. Despite the small differences between methods, this study confirms that the vast majority of fasted serum folate is present as 5-methyl-THF.

For urine and breastmilk samples, MA concentrations were consistently higher than the sum of 5-methyl-THF + FA measured via LC-MS/MS indicating the existence of additional folate species. Although breastmilk samples were incubated for 5 hours with an excess of folate conjugase (2 mL conjugase per 4 mL milk), it is possible that polyglutamate 5-methyl-THF remained in appreciable amounts. In an attempt to reconcile breastmilk MA and LC-MS/MS measurements, 5-methyl-THF-diglutamate and 5-formyl-THF were monitored for during LC-MS/MS quantification; however, neither was found in quantifiable amounts (data not shown). Thus, it is possible that some amount of 5-methyl-THF remained in polyglutamate form after trienzyme digestion and/or a larger volume of breastmilk (>4 mL) must be digested in order to detect and quantify other breastmilk folate species via this LC-MS/MS

method. Interestingly, preliminary results indicate 5-methyl-THF-diglutamate is present in quantifiable amounts in urine.

Conclusions

As compared using the Bland-Altman LoA, the sum of 5-methyl-THF and FA measured via LC-MS/MS is comparable to total folate measured via MA in fasted serum samples. Greater total folate measured via MA in urine and breastmilk indicate the existence of additional folate species. Recommendations for improving the agreement of MA and LC-MS/MS methods in urine and breastmilk include monitoring of 5-methyl-diglutamate in urine samples and extracting folate from > 4mL breastmilk for improved LC-MS/MS sensitivity.

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AFTERWORD

The overarching aim of this research was to elucidate aspects of folate and choline metabolism and utilization for the refinement of intake guidelines and strategies. Highlighted findings along with potential applications of this research are discussed below

Project 1: *Folate status response to a controlled folate intake among nonpregnant, pregnant, and lactating women*

Result highlights with implications and applications

The findings of this study have several implications for the advancement of folate intake guidelines and strategies. First, consumption of a FA-containing prenatal supplement plus normal mixed diet yielded supranutritional folate status, i.e. high serum folate concentrations and urinary folate excretion representing 9–43% of total folate intake, regardless of physiologic state. Notably, the increased utilization of /demand for folate during pregnancy and lactation was evident only in urinary folate excretion which was reduced in these physiologic states. In the current context of population wide folate sufficiency, this research will be useful for future folate DRI committees in considering whether the definition of adequacy should include the prevention of excess folate excretion/loss as is the case for the Vitamin C DRIs.

Perhaps the most important finding with implications for refinement of folate intake guidelines and strategies is that consumption of the study folate dose increased breastmilk FA concentrations but not total folate concentrations among participants that were not consuming a supplement prior to study enrollment. This result suggests that consumption of a FA-containing supplement (with underlying supranutritional

status) leads to the displacement of physiologic forms of folate from breastmilk by FA, which may be a less bioavailable folate form when bound to the human milk folate binding protein. Thus this research, in addition to informing the quantity of folate in prenatal supplements, indicates that the form of folate (i.e. FA vs. 5-methyl-THF) used in prenatal supplements and/or supplements produced for women of reproductive age should be revisited.

Project 2: *The influence of choline intake and pregnancy on phosphatidylcholine docosahexaenoic acid enrichment among third-trimester pregnant and nonpregnant women*

Result highlights with implications and applications

The results of this study have implications for both choline and DHA intake recommendations. First, among nonpregnant women, a higher choline intake yielded greater erythrocyte PC-DHA and a faster increase in plasma PC-DHA. Thus, the relationship between choline intake and erythrocyte and plasma PC-DHA enrichment among nonpregnant women in this study warrants the use of PC-DHA as a functional indicator of choline metabolism in this population. Second, third-trimester pregnancy was associated with alterations in choline related lipid metabolism which indicated an upregulation of the SAM-dependant PEMT PC biosynthetic pathway during the third trimester and increased demand for methyl donors during pregnancy. Further research is needed to determine whether an increased choline intake may exert a similar effect in increasing PC-DHA among pregnant women during the first and second trimesters of pregnancy when hepatic lipid metabolism and PEMT activity may more closely resemble that of nonpregnant women. Overall, these findings (along with others from

this study) will provide important data for revising the choline DRIs as well as generate hypotheses that further elucidate choline utilization and requirement among women of reproductive age.

The role of the SAM dependant PEMT pathway in DHA status and metabolism is underscored by this research. Findings of this study suggest a synergistic relationship between choline intake and DHA supplementation in enhancing DHA status in nonpregnant women, a result that may also hold true for pregnant women early in gestation. Thus, research aimed at improving DHA intakes and status may also consider the role methyl donor intake and status play in supporting the distribution of DHA to peripheral tissues and fetus in nonpregnant and pregnant women.

APPENDIX A

Genetic Variation: Impact on folate (and choline) bioefficacy[1]

ABSTRACT

Folate and choline are water-soluble micronutrients that serve as methyl donors in the conversion of homocysteine to methionine. Inadequacy of these nutrients can disturb one-carbon metabolism as evidenced by alterations in circulating folate and/or plasma homocysteine. Among common genetic variants that reside in genes regulating folate absorptive and metabolic processes, homozygosity for the MTHFR 677C>T variant has consistently been shown to have robust effects on status markers. This paper will review the impact of genetic variants in folate-metabolizing genes on folate and choline bioefficacy. Nutrient-gene and gene-gene interactions will be considered along with the need to account for these genetic variants when updating dietary folate and choline recommendations.

[1] Published in the *International Journal for Vitamin and Nutrition Research,* 2010;80:319–29, see Appendix C for inclusion authorization.

INTRODUCTION

Elucidating how nutrients are digested, absorbed, and metabolized in the human body is a fundamental endeavor of nutritional science. Nutrient bioavailability is considered to be a function of absorptive and metabolic processes that are influenced by such factors as nutrient form, dietary intake, and individual genetic variation [1] [2]. Other concepts that fall under the broader umbrella of bioavailability, as summarized in Gregory JF [2], are:

- Bioaccessibility: fraction available for absorption after release from food matrix
- Bioconversion/bioefficacy: fraction of absorbed nutrient converted to the bio-active form
- Functional bioefficacy: fraction of nutrient that impacts a functional parameter

Folate, a water-soluble B-vitamin, is widely recognized for its role in preventing neural tube defects (NTDs), its inverse relationship with numerous chronic diseases (i.e. coronary heart disease, stroke, cancers, osteoporosis, and dementia) and its usefulness as a chemotherapeutic target. The health importance of choline, a "quasi" essential micronutrient commonly grouped with the B-vitamins, is less known; however its effects on fetal development and programming [3], non-alcoholic fatty liver [4] [5] [6], and its intermingling with folate are attracting attention.

Folate and choline intersect at the metabolic network involved in the transfer of one-carbon units, referred to as one-carbon metabolism, which is required for the biosynthesis of nucleotides, the interconversions of select amino acids, and cellular methylation [7]. Homocysteine, formed in the methionine cycle, is an indicator of

cellular methylation distress (when elevated) and is associated with a plethora of health derangements. Methyl groups from folate or the choline derivative, betaine, can be used to convert homocysteine to methionine. In subsequent reactions, the nutrient-derived methyl group can be donated by S-adenosylmethionine (SAM) to acceptor molecules with effects on lipid metabolism, neurotransmitter biosynthesis and degradation, energy homeostasis, and gene expression and stability (i.e., DNA and histone methylation) (**Figure A.1**). Phosphatidylethanolamine *N*-methyltransferase (PEMT), a main consumer of SAM, catalyzes the sequential methylation of phosphatidylethanolamine to phosphatidylcholine (PtdCho) and provides a route for de novo synthesis of choline following its hydrolysis from PtdCho. As *S*-adenosylhomocysteine is a product of all SAM-dependent methyltransferases, the PEMT pathway is also a main producer of homocysteine (Figure A.1).

Folate bioefficacy can be assessed by measurements of serum/plasma and RBC folate concentrations, well-established markers of folate status [8]. 5-methyl-tetrahydrofolate (THF) is the main folate form in blood and its polyglutamated derivative is the principal bioactive form in tissue. Under normal physiologic conditions, circulating folate concentrations reflect the fraction of absorbed nutrient that is available to tissue and that can be converted to bioactive co-enzymes. Plasma total homocysteine (tHcy) concentration reflects folate availability for homocysteine remethylation to methionine and thus may be considered an indicator of functional folate bioefficacy [9].

Driven by the goal of discovering genetic polymorphisms that impact NTD development, homocysteine disposal, and the toxicity/efficacy of antifolate drugs,

genetic variation within folate-metabolizing genes has been widely studied over the past decade. From this body of literature, and more targeted studies, the impact of candidate genes and their polymorphisms on folate (and choline) bioefficacy can be examined. This paper provides examples of polymorphisms in folate-metabolizing genes that can impact folate (and choline) bioefficacy and explores the need to consider these variants when updating dietary recommendations.

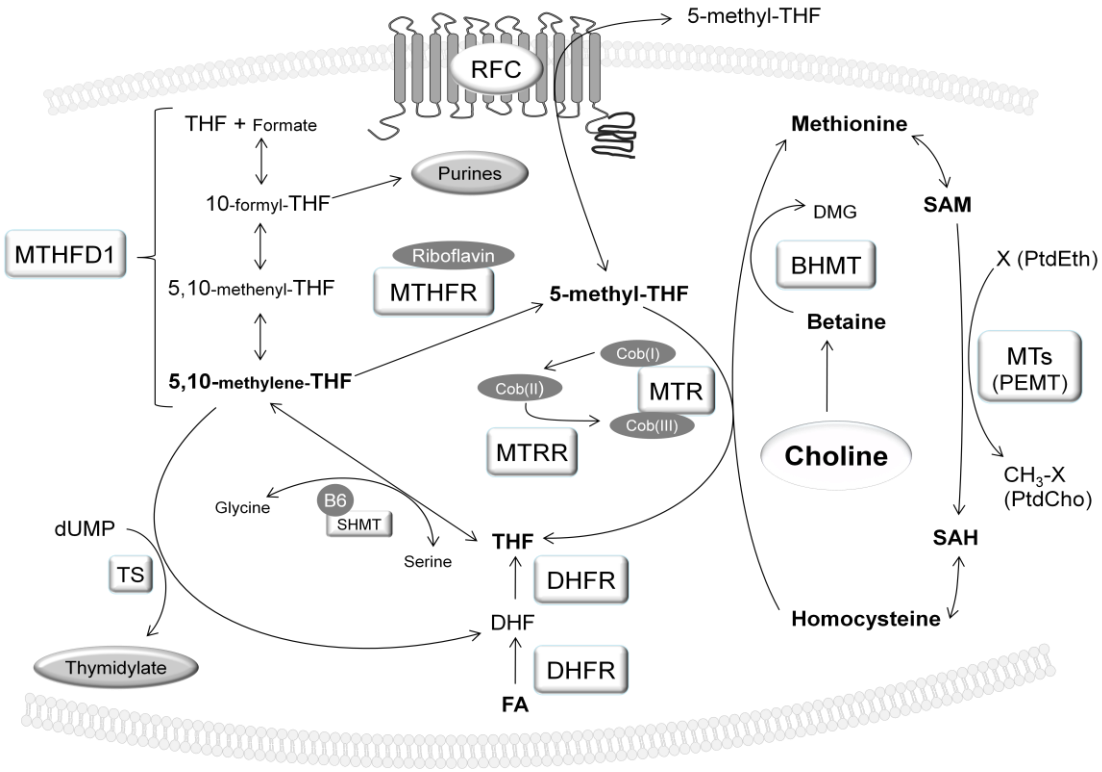

Figure A.1: Folate-metabolizing genes, with common genetic variants, that may impact folate (and choline) bioefficacy. Abbreviations: BHMT- betaine:homocysteine methyltransferases; Cob(I)- cob(I)alamin; Cob(II)- cob(II)alamin; Cob(III)-methylcob(III)alamin; DHF- dihydrofolate; DHFR- dihydrofolate reductase; DMG-dimethylglycine; dUMP- deoxyuridine monophosphate; FA- folic acid; MT- generic methyltransferases; MTHFD1- methylenetetrahydrofolate dehydrogenase; MTHFR-5,10-methylenetetrahydrofolate reductase; MTR- methionine synthase; MTRR-methionine synthase reductase; PEMT- phosphatidylethanolamine *N*-methyltransferase; PtEth- phosphatidylethanolamine; PtCho- phosphatidylcholine; RFC- reduced folate carrier; SAH- S-adenosyl homocysteine; SAM- S-adenosyl methionine; SHMT- serine hydroxymethyltransferase; THF- tetrahydrofolate; TS-thymidylate synthase

CANDIDATE GENES AND THEIR VARIANTS

Methylenetetrahydrofolate reductase (MTHFR)

MTHFR catalyzes the irreversible reduction of 5,10-methylene-THF to 5-methyl –THF (Figure A.1) and thereby plays a critical role in regulating one-carbon flux between nucleotide biosynthesis and cellular methylation. Moreover, as the enzyme producing the main form of folate in blood, MTHFR directly impacts circulating folate concentrations. The 677C>T variant allele in the MTHFR gene has garnered prodigy-like status over the past decade with approximately 300 human papers published per year on this single nucleotide polymorphism (SNP) (or ~ 75% of all publications on variants of genes in folate metabolism) [10]. In addition to having robust independent effects on folate status and health biomarkers, the MTHFR variant 677T allele frequently interacts with other one-carbon metabolizing SNPs to affect folate and choline status biomarkers.

MTHFR 677C>T

Discovered in 1995, this non-synonymous exonic SNP (rs1801133) is the most common genetic cause of mild hyperhomocysteinemia and is associated with a 35 or 65% loss in enzyme activity when one or two T alleles are present respectively [11] [12]. The 677C>T polymorphism causes a substitution of valine for alanine in the protein's catalytic domain and yields a thermolabile enzyme that is more likely to dissociate from its riboflavin derived FAD cofactor [13] [14]. The likelihood of having one or two copies of the 677C>T allele varies by ethnicity and geographic location [15] [16]. Within the United States, the prevalence of 677TT genotype averages 11% but is approximately 20, 12, and 1% among Hispanics, whites, and blacks,

respectively [17]. Worldwide, European, Asian, and African population prevalence of the 677TT genotype ranges from 4-20, 12- 30, and 0-4%, respectively [18]. Epidemiologic and controlled experimental studies consistently demonstrate lower folate status among individuals with the 677TT genotype especially when folate status is compromised.

NHANES III data that pre-dates folic acid fortification of the food supply in the United States reported 22% lower serum folate and 25% higher tHcy in those with the 677TT versus 677CC genotype [17]. Among an adult Danish population, the 677TT genotype increased the likelihood of low serum folate by over two times (<6.8 nmol/L; OR 2.24, P<0.001) [19]. Similarly, in an adult Norwegian population, the 677TT genotype is associated with a 32% increase in tHcy, a 29% decrease in serum folate, and a 10% reduction in the choline derivatives, betaine and dimethylglycine as compared to the 677CC genotype [20]. Although homozygosity for the 677T allele exerts the strongest effect on folate biomarkers, even a single copy of the 677T allele is associated with reduced folate status [17] [19] [20].

Highly controlled dose-response intervention studies have demonstrated a higher folate requirement in adults with the MTHFR 677TT genotype. Serum folate concentrations in Hispanic premenopausal women with the 677TT genotype were significantly lower throughout folate depletion (135 μg/day as dietary folate equivalents, DFEs) and during folate repletion with 400 μg DFE/day compared to those with the 677CC genotype [21]. These results paralleled a similar study conducted in non-Hispanic premenopausal women in which the 677TT genotype resulted in lower serum folate (pre- and post-repletion) and lower post-repletion RBC

folate [22]. In men, consumption of the folate RDA, 400 μg DFE/day, for 12 weeks yielded significantly lower serum folate and three times higher plasma tHcy in the 677TT versus the 677CC genotype [23].

The effects of genetic polymorphisms on folate bioefficacy can interact with nutrient intakes of folate, choline, vitamin B12, vitamin B6, and/or riboflavin [24] [25] [26]. The most striking example is in the case of MTHFR 677C>T and folate. Although lower serum folate concentrations were observed in women with the MTHFR 677TT genotype consuming either 135 or 400 μg DFE/day as discussed above [21], no differences in serum folate were detected between MTHFR C677T genotypes in another treatment arm consuming 800 μg DFE/day. Other studies have also shown that with an intake of ≥600 μg DFE/day, serum folate concentrations did not significantly differ between the MTHFR C677T genotypes [27] [28].

MTHFR 1298A>C

A second common non-synonymous SNP in the MTHFR gene, 1298A>C (rs1801131), causes the substitution of an alanine for glutamate in the regulatory domain of the MTHFR protein [29] [30]. The 1298A>C variant has been shown to reduce MTHFR enzyme activity in human lymphocytes; however, not to the extent 677C>T diminishes MTHFR activity [29] [30]. Notably, experiments using bacterial, mammalian, and yeast expression systems have failed to observe a functional impact of the 1298A>C SNP [14] [31] [26]. The prevalence of the 1298A>C allele varies by geographic region and ethnicity with 10% of whites and ~3.5% in blacks and Hispanics having the 1298CC genotype in the US [17]. Among European, Asian, and

African populations, prevalence of the 1298CC genotype ranges from 4-13, 2-5, and 0-1%, respectively [18].

The impact of the MTHFR1298A>C SNP on folate status is ambiguous. The majority of studies, including the NHANES III study described above, have found no significant associations between the 1298A>C variant and markers of folate status [17] [19] [30]. However, a large scale epidemiologic study found that compared with the homozygous wild type genotype, the 1298CC genotype was associated with a 5% increase of tHcy and a decrease of 9% and 3% for serum folate and betaine, respectively [20]. Thus, while there is evidence that the 1298CC genotype impacts folate bioefficacy, the current body of evidence indicates that the MTHFR 677TT genotype has a much greater effect on both metabolic bioefficacy, (i.e. generation of 5-methyl-THF) and functional bioefficacy (i.e. plasma tHcy concentrations).

MTHFR Haplotypes: 677C>T + 1298A>C

The 677C>T and 1298A>C SNPs are rarely found on the same allele (i.e. these SNPs are in strong linkage disequilibrium) [30] [32] which implies that the metabolic impact of the MTHFR A1298C genotype should be considered within the context of the MTHFR C677T genotype. A MTHFR genotype meta-analysis calculated Caucasian population MTHFR haplotype frequencies as follows: 677C/1298A, 37%; 677C/1298C, 31%; 677T/1298A, 32%; and 677T/1298C, 0.23-0.34% [32]. Notably, heterozygous MTHFR genotypes are of interest due to widespread prevalence and evidence of functional consequences (i.e., penetrance). The 677CT plus 1298AC genotype is associated with reduced MTHFR enzyme activity, reduced plasma folate, and increased tHcy [29] [30] [33]. A large scale epidemiologic study (n=10,034)

ordered MTHFR genotypes by decreasing levels of serum folate as follows: 677CC/1298AA (highest) > 677CC/1298AC > 677CC/1298CC ~ 677CT/1298AA > 677CT/1298AC > 677TT/1298AA (lowest) [33]. After comparing these genotypes with a model that included six MTHFR protein configurations of varying functionality and stability, the investigators found that the least functional/stable enzyme configurations corresponded with particular genotypes and lower serum folate concentrations [33]. These results indicate that although the effect of the heterozygosity for the 1298A>C and 677C>T SNPs do not always emerge as significant predictors of status endpoints, depending on genotype and folate exposure, these genetic polymorphisms can have an important impact on MTHFR enzyme function and folate bioefficacy.

Reduced Folate Carrier (RFC)

RFC, the product of the SLC19A1 gene, is a bidirectional folate transporter that is ubiquitously expressed and represents a main route of folate entry into most systemic tissues. However, despite being expressed in enterocytes, RFC does not appear to play a significant role in intestinal folate absorption. Instead, absorption of folate across the intestinal epithelium is mediated by the more recently identified proton-coupled folate transporter (PCFT) [34]. Mutations in PCFT are a cause of hereditary folate malabsorption [35]; however, putative common PCFT genetic variants have not yet been reported.

RFC spans the plasma membrane 12 times and has a relatively high affinity for reduced folates, including 5-methyl-THF, but a low affinity for oxidized synthetic folic acid. A common SNP resides at position 80 in exon 2 of RFC which

changes a guanine to adenine (RFC 80G>A; rs1051266); although 80A>G is also reported [18] [36]. The 80G>A SNP causes a substitution of histidine for arginine and is predicted to reside in the first transmembrane domain, a region implicated as important to carrier function [37]. Among European, Asian, and African populations, prevalence of the 80GG genotype has been reported to be ~29, ranging from 20-30, and ~13%, respectively [18]. Existing data show null [36] [20] or nominal [38] effects of the RFC 80AA genotype on markers of folate status including serum folate, RBC folate and plasma homocysteine. A study conducted in Ireland reported higher RBC folate in women homozygous for the variant 80A allele; nevertheless, the genotype explained only a minor percentage, 5%, of RBC folate variation [38]. Some studies report an interaction between the RFC G80A and MTHFR C677T genotypes [36] [39] [40] but due to discrepancies in nomenclature, the relationship of the interaction is unclear. Like MTHFR 1298A>C and MTRR 66A>G (see next section), RFC 80G>A SNP in isolation does not appear to have a strong effect but may synergistically interact with the MTHFR 677C>T SNP to impact folate bioefficacy.

MTR & MTRR

Upon cellular entry, THF is generated from 5-methyl-THF when it is used to convert homocysteine to methionine in a reaction catalyzed by the vitamin B-12 (cobalamin) dependent methionine synthase (MTR) (Figure A.1). MTR-bound cob(I)alamin can be methylated by 5-methyl-THF to generate the methylcob(III)alamin intermediate which serves as the direct methyl donor in conversion of homocysteine to methionine. Alternatively, MTR-bound cob(I)alamin can be oxidized to the nonfunctional cob(II)alamin form which is subsequently

reduced and methylated to the methylcob(III)alamin intermediate via the action of SAM dependent methionine synthase reductase (MTRR) [41].

Genetic variation in MTR and MTRR has the potential to interfere with the production of methionine and THF thereby disrupting folate homeostasis and elevating tHcy [42]. The MTR 2756A>G SNP (rs1805087) results in a glycine for aspartic acid substitution and appears to have a potentially protective effect as it is associated with reduced tHcy concentrations [20] [42] [43], an effect that is independent of the MTHFR C677T genotype. Conversely, the nonsynonymous MTRR 66A>G SNP (rs1801394) is associated with increased tHcy but only when combined with the MTHFR 677TT genotype [17] [43] [44]. Given the high prevalence of the MTR 2756G and MTRR 66G variant alleles, 19 and 25-60% [17] [20], these SNPs have the potential to impact folate bioefficacy in a large number of individuals.

DHFR

Dihydrofolate, a product of thymidylate synthase, is reduced to THF by the enzyme dihydrofolate reductase (DHFR) (Figure A.1). Thus, DHFR plays a critical role in maintaining the cellular supply of reduced folates required for DNA synthesis. Folic acid is an additional substrate of DHFR, which via two consecutive reductions, enters the folate cycle as THF. Thus, DHFR is essential for converting folic acid, the synthetic folate used in food supply fortification and vitamin supplements, into a form that is usable by the body. Given these two functions, polymorphisms in the DHFR gene are of considerable interest in the folate bioefficacy discussion.

To date, there have been no SNPs identified in the coding region of DHFR; however, a 19 base pair deletion (19-bp del) polymorphism in intron 1 of the DHFR

promoter (reference sequence NC_000005.8) results in the loss of a transcription factor binding site which may alter protein expression [45]. The 19-bp deletion allele is common with measures of the deletion/deletion (del/del) genotype of ~ 17-33% [45] [46] [47] [48]. The 19-bp del polymorphism appears to be functional, however, it is unclear whether gene expression is increased [47] [49] or decreased [45] [50].

Reports of the association between the 19-bp del variant and folate status have also been mixed. One study reported that the homozygous 19-bp del/del and heterozygous 19-bp del/wild type genotypes were associated with 14.4 and 2.5% reductions in tHcy [46]. Another study that seems to support the former, found that among young Irish women the homozygous 19-bp del/del genotype was marginally associated with increased RBC and serum folate levels [51]. A third study found interactions between the 19-bp del/del genotype, folic acid dose, RBC folate, and circulating un-metabolized folic acid [48]. The homozygous 19-bp del/del genotype was associated with lower RBC folate on a folic acid dose of <250 ug/d, but higher plasma un-metabolized folic acid with a dose of ≥500 ug/d [48]. These findings suggest impaired functional capacity of the 19-bp del DHFR enzyme to convert folic acid to a physiologic form.

Genetic variation in DHFR may contribute to the nearly 5-fold variation in DHFR enzyme activity measured in human liver samples (n=6) [52]. Of particular note in discussing bioconversion is that the reduction reaction involving synthetic folic acid and DHFR was significantly slower than the reduction reaction involving physiologic dihydrofolate and DHFR [52]. Although further research is warranted,

these findings suggest that the 19-bp del DHFR variant, and perhaps other DHFR variants, have the potential to impact folate bioefficacy.

GENETIC POLYMORPHISMS AND CHOLINE BIOAVAILABILITY

The interplay of folate and choline in one-carbon metabolism has been well described [7] and recently highlighted by genetic variants in folate-metabolizing genes altering biomarkers of choline status.

Methylenetetrahydrofolate dehydrogenase (MTHFD1)

The MTHFD1 gene codes for a tri-functional enzyme that enables entry of mitochondrial one-carbons into the cytosol with its 10-formylTHF synthetase activity and facilitates the interconversions of 10-formylTHF, 5,10-methenylTHF and 5,10-methyleneTHF with its cyclohydrolase and dehydrogenase activities [53] (Figure A.1). A non-synonymous guanine to adenine SNP at nucleotide 1958 prompts the substitution of a glutamine for arginine amino acid in the 10-formylTHF synthetase domain (MTHFD1 1958G>A; rs2236225) [54]. Prevalence of the variant MTHFD1 1958AA genotype is 13-22, 0-9, and 3-5% in European, Asian, and African populations, respectively [18].

Although the 1958G>A SNP has not been associated with impaired folate status in epidemiological studies [20] [54], one or two copies of the variant 1958A allele increases the risk of exhibiting signs of choline deficiency (i.e. organ dysfunction) in response to dietary choline depletion (OR=7, P=0.007) [55]. This risk is further enhanced if the choline deficient diet is coupled with a low folate intake. Premenopausal women carrying one or two copies of the variant 1958A allele were especially susceptible to increased risk of organ dysfunction under these dietary regimens (OR=85, P<0.0001). The investigators hypothesized that the variant 1958A allele results in a dysfunctional MTHFD1 enzyme which reduces the flow of folate

one-carbon units towards homocysteine remethylation. This in turn places a greater burden on one-carbon units derived from the choline metabolite betaine, leading to choline deficiency. Additionally, it may reduce the pool of SAM that is available for *denovo* biosynthesis of PtdCho through the PEMT pathway. PEMT is upregulated by estrogen which enables premenopausal women to make more choline endogenously (relative to men and postmenopausal women) [57] [8]. Thus, a dysfunctional MTHFD1 may impair the PEMT reaction and contribute to the excess susceptibility of premenopausal women with the variant 1958A allele to choline depletion.

The tendency for tHcy to increase more in premenopausal women if they had the MTHFD1 1958AA genotype [relative to the 1958GA (P=0.033) and 1958GG (P=0.085) genotypes supports the hypothesis that the 1958G>A SNP has a functional effect on cellular methylation [56]. It should be noted that this effect was only observed on a folate restricted diet and disappeared with subsequent folate repletion [56]. In vitro studies show the MTHFD1 1958A>G protein is thermolabile with reduced enzyme activity; however, added folate restores the 1958A>G protein to wild type enzyme activity levels [58]. Collectively, these findings indicate that under conditions of sub-optimal folate/choline intake, the MTHFD1 1958A>G SNP impacts folate and choline bioefficacy by modulating the availability of 5-methyl-THF.

MTHFR

Several studies have shown that MTHFR deficiency alters the metabolism of choline [59] [60] [61] [62]. In young men consuming sub-optimal folate intakes along with one of four choline intakes (range: 300-2200 mg/d), lower concentrations of plasma PtdCho were observed in the 677TT versus CC genotype regardless of choline

intake [60]. Diminished PtdCho in the MTHFR 677TT genotype under these dietary constraints may arise from increased use of choline as a methyl donor. This working hypothesis was recently supported by a tracer study involving a sub-sample of these men in which a greater flux of choline towards betaine (and other oxidative products) was detected in the MTHFR 677TT [63]. Although, the MTHFR C677T genotype modifies choline metabolism in premenopausal women, the relationship is less clear likely due to engagement of compensatory mechanisms. For example, in premenopausal women that completed a study with constant choline intake of 349 mg/d and folate depletion and repletion stages ([21] discussed above), plasma PtdCho decreased significantly during folate depletion, however, women with the MTHFR 677TT genotype tended to be resistant to the decline (P=0.089) [59]. When serum folate was included as a covariate in the statistical model, MTHFR genotype emerged as a predictor of plasma PtdCho (P=0.027) and PtdCho was significantly higher in women with the 677TT genotype (P=0.032) [59].

DISCUSSION

Genetic variants in folate metabolizing genes have been associated with risk of a variety of diseases, including birth defects, cancer, and cardiovascular disease. Mechanisms and causation are yet to be proven; however, proposed pathways involving DNA stability and one-carbon partitioning have been suggested [64]. Implicit in genetic variation and disease risk associations is that genetic polymorphisms alter nutrient bioefficacy such that protein and cellular functions are altered, ultimately conferring either increased or attenuated disease risk. Although there is debate about what can be concluded from associations between common genetic polymorphisms in folate-metabolizing genes and disease risk [65] [66], stronger conclusions about the impact of genetic polymorphisms on folate bioefficacy can be made.

Homozygosity for the MTHFR 677C>T variant allele is the most robust modifier of folate status markers among the folate-metabolizing SNPs investigated thus far. However, the severity of 677TT genotype on markers of folate metabolism depends to a large degree on folate intake because relatively high intracellular concentrations of folate can stabilize the functional impairment caused by the 677C>T SNP. Due mainly to folic acid fortification of the food supply, the average folate intake for men and women in the United States is 813 and 724 μg DFE/day, respectively [67], which is well above the current US folate RDA for adults (400 μg DFE/day) and above the level at which strong effects of the 677TT genotype are observed (<600 μg DFE/day). Thus, given current folate intakes in the US (and other fortified populations) along with the marginal effects of common genetic variation on

folate and choline bioefficacy (reviewed in part herein), it is unlikely that SNPs in folate-metabolizing genes, even the MTHFR 677C>T, detrimentally impact folate and choline status at the population level. A US epidemiological study examining serum folate and tHcy levels pre- and post-folic acid fortification stratified by MTHFR C677T genotype reveals as much: those with the 677TT genotype have significantly higher tHcy at both time points, however, the difference in means goes from 2.5 μmol/L pre-fortification to ≤0.7 μmol/L post-fortification [68].

Although there is little evidence that population level folate status is compromised by common genetic polymorphisms in folate-metabolizing genes in the US, the issue of whether dietary recommendations should consider genetic variation remains [69] [70]. Research examining the current RDA of 400 μg DFE/day has shown it to be adequate for women with the 677TT genotype [21] [22], yet insufficient for men with the 677TT genotype [23]. In a recent study, 34% and 79% of men with the 677TT genotype had serum folate concentrations <6.8 nmol/L and tHcy >14 μmol/L after 12 weeks of consuming the folate RDA [23]. Interestingly, these same signs of folate deficiency were exhibited in 16% and 7% of men with the 677CC genotype [23]. This small, yet highly controlled study raises uncertainty as to whether the current folate RDA, 400μg DFE/day, is sufficient for meeting the requirement of 97% of men. Using a model that projected folate RDA ranges based on the 677TT genotype and different functional effect sizes (5-50%), Robitaille et al. [71] concluded that the MTHFR 677TT genotype does not warrant changes to the 1998 folate RDA. This type of statistical approach in assessing whether common SNPs affect nutrient requirements is highly useful but in this particular case [71], the adequacy of the folate

RDA for the MTHFR 677CC genotype is questionable given the results of a metabolic study in which folate intake was controlled [23].

It has long been recognized that folate metabolism is under genetic control and that genetic heterogeneity explains a large percentage (i.e. > 40%) of variation in metabolism [8]. Historically, this genetic heterogeneity has been accounted for when setting RDAs by adding two coefficients of variation (CV) of 10 percent each to the estimated average intake (i.e. EAR x 1.2). However, the assumption of a 10 percent CV is based on variation in basal metabolic rate [8] and may not adequately capture the impact of genetic variation in folate-metabolizing genes on bioefficacy and requirements. The plethora of studies published over the past decade on SNPs and aspects of folate metabolism afford the opportunity to refine estimates of variability such that effects of genetic background (as well as other factors) could be more thoroughly addressed. This type of analysis may in turn alleviate the need to individualize dietary recommendations on the basis of genetic sub-groups.

CONCLUSION

The impact of genetic variation on folate and choline status markers is well-studied. Homozygosity for the variant MTHFR 677T allele is a strong modifier of folate (and choline) bioefficacy particularly under conditions of sub-optimal folate intake. Gene-gene interactions are also apparent such that certain combinations (i.e. MTHFR 677TT and RFC 80GG) may exacerbate metabolic disturbances. A nutrient specific estimate of population wide variability in folate bioefficacy would provide a background on which to determine the need to account for functional SNPs (within the

context of complex interactions) in revising dietary recommendations for folate and choline.

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APPENDIX B: Feeding study documents

Pregnant Women
Health History Questionnaire

Demographic Information

Date: _____
Month Day Year

Name: _____
First Last

Phone _____ _____ _____
Home Cell Email

Address:

_____

_____

_____

Age: _____ Date of Birth: _____
Mo. Day Year

Education Completed:

_____High School Graduate _____Master's Degree

_____Bachelor's Degree _____Doctoral Degree

Present Work/Student Status:

_____Working Full-time _____Full-time student
_____Working Part-time _____Part-time student
_____Not Employed _____Retired

Pregnancy Information

Gestational Age at Present: _____ (weeks)

Date of Last Menstrual Period: _____ (month/day/year)

Due Date: _____ (month/day/year)

Anthropometric Data

Height (without shoes) _____

Pre-Pregnancy weight (dressed with shoes) _____

Current weight (dressed with shoes) _____

Medical History

Indicate if you have had or currently have any of the following medical problems. Check all that apply:

Yes	No	
___	___	Alcoholism
___	___	Cardiovascular Disease (Atherosclerosis / Heart Attack/Stroke)
___	___	Eye Problems (ie, glaucoma, cataracts, retinopathy)
___	___	Gall Bladder Disease
___	___	High Blood Cholesterol
___	___	High Blood Pressure
___	___	Intestinal Disorders
___	___	Kidney Disease
___	___	Liver Disease
___	___	Lung Disease

___ ___ Neurologic/Seizure Disorder

___ ___ Obesity / Overweight

___ ___ Thyroid Disease

___ ___ Tumors / Cancer – List type: _____

___ ___ Ulcers

___ ___ Other – Specify: _____

___ ___ Recent surgeries? If yes, specify: _____

Indicate the prescription/nonprescription medicines you currently use on a regular basis.
Check all that apply:

___ Allergy Medicines/Antihistamines ___ Antacids

___ Antibiotics ___ Anti-arrhythmics

___ Anti-inflammatory Agents (i.e. Ibuprofen) ___ Aspirin

___ Asthma Medicines ___ Beta Blockers

___ Blood Pressure Medicines ___ Blood Thinners

___ Diabetes Medicines – Specify: _____

___ Diuretics ___ Gout Medicines

___ Heart Medicines – Specify: _____

___ Hormones – Specify: _____

___ Seizure Medicines ___ Thyroid Medicines

___ Medicines interfering with folate metabolism – Specify: _____

___ Other – Specify: _____

Supplement Use

Indicate the supplements you currently use on a regular basis.
Check all that apply.

___ Prenatal Supplement (name/brand)
_____

___ Multivitamin/Minerals ___ Herbs

___ Amino acids ___ Ergogenic Aids (i.e., creatine)

___ Other-Specify: _____

Activity Status

What would best describe your activity level?

____ Level 1: Usual daily activities
____ Level 2: Some form of exercise (i.e., walking, dancing, riding a bike) at least 3 times per week
____ Level 3: Vigorous exercise (i.e., singles tennis, running, aerobics, weight lifting) at least 3 times/ week.

Beverage Consumption

Indicate the amount and frequency with which you consume the following at present:

	Amt. (each)	Never/Rarely (5x/yr)	Occasionally (2-3x/mo.)	Frequently (2-3x/wk.)	Daily
Beer	_____	_____	_____	_____	_____
Liquor	_____	_____	_____	_____	_____
Wine	_____	_____	_____	_____	_____
Wine Coolers	_____	_____	_____	_____	_____
Coffee	_____	_____	_____	_____	_____
Tea	_____	_____	_____	_____	_____
Soda	_____	_____	_____	_____	_____

Lactating Women
Health History Questionnaire

Demographic Information

Date: _____
Month Day Year

Name: _____
First Last

Phone _____ _____ _____
Home Cell Email

Address:

_____

_____

_____

Age: _____ Date of Birth: _____
Mo. Day Year

Education Completed:

_____High School Graduate _____Master's Degree

_____Bachelor's Degree _____Doctoral Degree

Present Work/Student Status:

_____Working Full-time _____Full-time student
_____Working Part-time _____Part-time student
_____Not Employed _____Retired

Pregnancy Information

Due Date: _____ (month/day/year)

Delivery Date: _____ (month/day/year)

Health Insurance Provider: _____

Total Weight Gained During Pregnancy: _____

Complications During Pregnancy: _____ Yes _____No

If yes, describe:

_____
_____
_____
_____

Complications During Labor: _____Yes _____No

If yes, describe:

_____
_____
_____
_____

Newborn Information

Date of Delivery: _____
Month Day Year

Mode of Delivery (i.e., vaginal or C-section): _____

Gestational Age: _____

Length: _____

Weight: _____

Head Circumference: _____

Anthropometric Data

Height (without shoes) _____

Pre-Pregnancy weight (dressed with shoes) _____

Current weight (dressed with shoes) _____

Medical History

Indicate if you have had or currently have any of the following medical problems. Check all that apply:

Yes	No	
___	___	Alcoholism
___	___	Cardiovascular Disease (Atherosclerosis / Heart Attack/Stroke)
___	___	Eye Problems (ie, glaucoma, cataracts, retinopathy)
___	___	Gall Bladder Disease
___	___	High Blood Cholesterol
___	___	High Blood Pressure
___	___	Intestinal Disorders
___	___	Kidney Disease
___	___	Liver Disease
___	___	Lung Disease
___	___	Neurologic/Seizure Disorder
___	___	Obesity / Overweight
___	___	Thyroid Disease
___	___	Tumors / Cancer – List type: _____

___ ___ Ulcers

___ ___ Other – Specify: _____

___ ___ Recent surgeries? If yes, specify: _____

Indicate the prescription/nonprescription medicines you currently use on a regular basis.
Check all that apply:

___ Allergy Medicines/Antihistamines ___ Antacids

___ Antibiotics ___ Anti-arrhythmics

___ Anti-inflammatory Agents (i.e. Ibuprofen) ___ Aspirin

___ Asthma Medicines ___ Beta Blockers

___ Blood Pressure Medicines ___ Blood Thinners

___ Diabetes Medicines – Specify: _____

___ Diuretics ___ Gout Medicines

___ Heart Medicines – Specify: _____

___ Hormones (ie, birth control bills) – Specify:
_____

___ Seizure Medicines ___ Thyroid Medicines

___ Medicines interfering with folate metabolism – Specify: _____

___ Other – Specify: _____

Supplement Use

Indicate the supplements you currently use on a regular basis.
Check all that apply.

___ Prenatal Supplement (name/brand) _____

___ Multivitamin/Minerals ___ Herbs

___ Amino acids ___ Ergogenic Aids (i.e., creatine)

___ Other-Specify: _____

Activity Status

What would best describe your activity level?

____ Level 1: Usual daily activities
____ Level 2: Some form of exercise (i.e., walking, dancing, riding a bike) at least 3 times per week
____ Level 3: Vigorous exercise (i.e., singles tennis, running, aerobics, weight lifting) at least 3 times/ week.

InfoNon-Pregnant Women
Health History Questionnaire

Demographic Information

Date: _____
Month Day Year

Name: _____
First Last

Phone _____ _____ _____
Home Cell Email

Address:

_____

_____

_____

Age: _____ Date of Birth: _____
Mo. Day Year

Education Completed:

_____High School Graduate _____Master's Degree

_____Bachelor's Degree _____Doctoral Degree

Present Work/Student Status:

_____Working Full-time _____Full-time student
_____Working Part-time _____Part-time student
_____Not Employed _____Retired

Number of Children: _____

Number of pregnancies: _____

Anthropometric Data

Height (without shoes) _____

Current weight (dressed with shoes) _____

Medical History

Indicate if you have had or currently have any of the following medical problems. Check all that apply:

Yes	No	
___	___	Alcoholism
___	___	Cardiovascular Disease (Atherosclerosis / Heart Attack/Stroke)
___	___	Eye Problems (ie, glaucoma, cataracts, retinopathy)
___	___	Gall Bladder Disease
___	___	High Blood Cholesterol
___	___	High Blood Pressure
___	___	Intestinal Disorders
___	___	Kidney Disease
___	___	Liver Disease
___	___	Lung Disease
___	___	Neurologic/Seizure Disorder
___	___	Obesity / Overweight
___	___	Thyroid Disease
___	___	Tumors / Cancer – List type: _____
___	___	Ulcers
___	___	Other – Specify: _____
___	___	Recent surgeries? If yes, specify: _____

Indicate the prescription/nonprescription medicines you currently use on a regular basis.
Check all that apply:

___ Allergy Medicines/Antihistamines ___ Antacids

___ Antibiotics ___ Anti-arrhythmics

___ Anti-inflammatory Agents (i.e. Ibuprofen) ___ Aspirin

___ Asthma Medicines ___ Beta Blockers

___ Blood Pressure Medicines ___ Blood Thinners

___ Diabetes Medicines – Specify: _____

___ Diuretics ___ Gout Medicines

___ Heart Medicines – Specify: _____

___ Hormones – Specify: _____

___ Seizure Medicines ___ Thyroid Medicines

___ Medicines interfering with folate metabolism – Specify: _____

___ Other – Specify: _____

Supplement Use

Indicate the supplements you currently use on a regular basis.
Check all that apply.

___ Multivitamin/Minerals ___ Herbs

___ Amino acids ___ Ergogenic Aids (i.e., creatine)

___ Other-Specify: _____

Activity Status

What would best describe your activity level?

____ Level 1: Usual daily activities

____ Level 2: Some form of exercise (i.e., walking, dancing, riding a bike) at least 3 times per week

____ Level 3: Vigorous exercise (i.e., singles tennis, running, aerobics, weight lifting) at least 3 times/ week.

Beverage Consumption

Indicate the amount and frequency with which you consume the following:

	Amt. (each)	Never/Rarely (5x/yr)	Occasionally (2-3x/mo.)	Frequently (2-3x/wk.)	Daily
Beer	_____	_____	_____	_____	_____
Liquor	_____	_____	_____	_____	_____
Wine	_____	_____	_____	_____	_____
Wine Coolers	_____	_____	_____	_____	_____
Coffee	_____	_____	_____	_____	_____
Tea	_____	_____	_____	_____	_____
Soda	_____	_____	_____	_____	_____

Consent, Pregnant Women

Cornell University
Informed Consent for Research Involving Human Study Participants

You are being invited to participate in a research study. This form is designed to provide you with information about this study. The Principal Investigator or representative will describe this study to you and answer any of your questions.

Project Title:

Effect of maternal choline intake on maternal/fetal biomarkers of choline status

Investigators:

Marie Caudill, PhD, RD; Associate Professor, Division of Nutritional Sciences, Cornell University

Eva Pressman, MD. Director of Obstetrics and Maternal-Fetal Medicine, Department of Obstetrics and Gynecology, University of Rochester School of Medicine, Rochester, New York

Richard Canfield, PhD. Research Associate, Division of Nutritional Sciences, Cornell University

Barbara Strupp, PhD. Professor, Division of Nutritional Sciences, Cornell University

Srisatish Devapatla, MD. Director, Special Care Nursery, Chairman- Dept. of Pediatrics, Cayuga Medical Center at Ithaca, Ithaca, New York.

Sandy Saintonge, MD. Assistant Professor of Clinical Public Health and Clinical Pediatrics, Weill Cornell Medical College, Attending Physician, New York Hospital Queens, Department of Emergency Medicine

Cydne Perry, PhD. Post-doctoral Associate, Division of Nutritonal Sciences, Cornell University

What the study is about:

Choline, a nutrient found in the diet (i.e., eggs, nuts, and beef), was recently recognized as a required nutrient for humans. The recommended dietary intake level for pregnant women is 450 mg/d. However, at present, it is not known whether this amount is optimal for pregnant women. *Thus, this study seeks to assess the effect of pregnancy on choline status and to examine whether the current recommended dietary intake, 450 mg/d, is enough to optimize choline status during pregnancy.*

There is evidence from animal studies that excess choline consumed by the mother during pregnancy may have long lasting beneficial effects on the memory and attention of the newborn. However, no study has examined this in humans. *Thus, this study seeks to assess the effect of extra choline (450 mg/d) for a total choline intake of 900 mg/d during pregnancy on learning, attention and memory in the infant. The choline intake of 900 mg/d is an amount that can be obtained by the diet and is well below the upper level of tolerance, 3500 mg/d.*

There is evidence that choline status affects folate (another essential nutrient) and vice versa. *Thus, this study seeks to examine the relationship between choline and folate status.*

What we will ask you to do:

Screening Phase:

First, it is necessary to determine whether you meet the inclusion criteria of this study. This process will take up to 1 hour. The inclusion criteria are as follows:

- I am pregnant *(non-pregnant women are also eligible for the study in order to provide us with a control group)*
- I have not entered my third trimester (i.e., wk 27)
- I am between 21 and 40 years of age.
- I eat meat and other animal products
- I am not a current smoker.
- I am not using recreational drugs
- I am not drinking alcoholic beverages
- I am healthy
- I do not have gestational diabetes
- I do not have anemia.
- I do not have kidney or liver problems.
- I am not chronically taking medication that affects liver function.
- I had a body mass index (BMI) < 34 as my pre-pregnancy weight

During the screening phase, you will have your blood drawn (~ 4 tablespoons) which will be used to assess your health status and measure select vitamins/nutrients. DNA will also be obtained from your blood in order to investigate the effect of nutrients for people of different genetic make-up. You will also complete a health history questionnaire.

Dietary Intervention Phase

If you meet the required criteria, you will be invited to participate in a twelve week feeding study. Throughout this study you will consume a normal mixed diet consisting of breakfast, lunch, dinner and snacks. You will consume at least one meal per day (breakfast, lunch or dinner) in the human metabolic research unit located at Cornell University. The other meals will be provided as takeout meals. You will also consume a

prenatal vitamin/mineral supplement to ensure that you are meeting the recommended dietary intake levels for pregnant women of all the essential nutrients. In addition, you will be randomized to a choline supplement to achieve total choline intakes of 450 or 900 mg/d. To increase the study's validity, the level of choline intake to which you are assigned (i.e., 450 or 900 mg/d) will not be disclosed.

To help us better understand choline requirements and use during pregnancy, a small portion of the choline will be labeled from week 6 to 12. This label (marker) is safe and has been used many times in studies involving babies, young children and pregnant women.

It is important that you eat all of the food that we give you and that you eat nothing else outside of the study food. You will be weighed every week and extra food/beverages will be provided as necessary to ensure appropriate weight gain (~ 1 pound per week).

Blood will be taken by a trained phlebotomist (i.e., a person that is trained to draw blood) before the start of the study and at weeks 3, 6, 9, 10 and 12. The blood will be used to analyze how choline intake, folate intake and/or pregnancy influences biomarkers of status. Also we will use the DNA obtained from the blood to analyze how genetic differences influence the use of choline and/or folate. In addition, you will be required to collect your urine over a 24 hour period at the beginning of the study (week 0) and at weeks 6, 9, and 12.

Time Between End of Dietary Intervention Phase and Delivery

We request that you continue on the choline supplement that you consumed during the intervention study (ie, 100 or 500 mg/d) until you delivery the baby. This is an estimated time of 1 week. We will provide the choline supplement on a weekly basis.

At Delivery

We intend to obtain a final blood sample from you and from the umbilical cord when you deliver the baby. This blood will be used to measure your choline and folate status as well as that of the baby (i.e., umbilical cord). We will also use the cord blood to extract DNA to provide information on

the influence of genetic differences on choline/folate status in your baby. In addition, we intend to retain the placenta which is normally discarded after the delivery of your baby. Your placenta will be used to examine how choline intake influences the expression of relevant genes as well as choline utilization. Lastly, we intend to ask you a few questions (i.e., less than 5 minutes of your time) about your delivery and the baby's height and weight (included in your informational packet).

Samples obtained during your participation on this study may be used by the investigators (or collaborators) for the purposes of learning more about other nutrients (ie., vitamin D and biotin) and how genetic differences affect requirements for these nutrients.

It is possible that other investigators will have access to your blood samples (including DNA), placental tissue, and urine samples; however, the samples will be de-identified (ie, no names will be used).

Cognitive Testing of Infant

We would like to perform cognitive tests that assess your infant's attention and memory when he/she is three, six, nine and twelve months of age. These tests will measure your child's eye movements to changes in pictures/images. Feeding your baby between tests is fine. These tests are non-invasive. Each test is approximately 5-10 minute and will take up to 30 minutes total.

Risks and benefits:

We foresee minimal risks as part of your participation in this study. The choline dosage of 450 mg/d is the recommended amount for pregnant women and is considered to be adequate. The choline dosage of 900 mg/d is considered to be safe (Institute of Medicine, National Academy of Science) and falls within the range of choline intake consumed by the general population. *None-the-less, we will monitor your health status as you progress through this study. Specifically we will obtain a blood chemistry profile and a complete blood count at weeks 0, 3, 6, 9 and 12. These tests provide information on your blood lipids, liver and kidney function, and iron status. We will also take your blood pressure on a weekly basis. Should any problems be identified, we will refer you to your physician who will advise on your continuing to participate in this study.*

As with any blood draw, it is also possible that you will experience some dizziness, ill-feeling and bruising during/after the blood draw. In addition, having blood samples taken always includes a certain low risk of infection. *To minimize this risk, blood will be drawn by a trained phlebotomist at the human metabolic research unit while you are lying down.*

There are no established direct benefits to you or your baby. From this study we hope to draw conclusions regarding the optimal intake levels of choline and folate in pregnant women, potentially benefiting the health of other women and children.

In the event that you should be injured in the course of this research study, you will be provided with necessary medical care in Cayuga Medical Center At Ithaca. This statement does not mean that either such medical care or hospitalization, if necessary, will be free of charge.

Compensation:

Week of Study	Payment
1	$50
2	$60
3	$70
4	$80
5	$100

6	$120
7	$140
8	$160
9	$180
10	$200
11	$220
12	$240

You will be paid the dollar amounts indicated above in two allotments ($480 at week 6 and $1140 at week 12) for a total of $1620. Should you stop participating in the study, you will be compensated for the time you have spent on the study.

You will also be compensated for the blood and information obtained at delivery ($100) and for each cognitive evaluation session ($50 per session x 4 sessions for a total of $200).

Taking part is voluntary:

Your participation in this study is completely voluntary. If you decide to take part, you are free to withdraw at any time; however you will not be eligible to receive monetary compensation after ending the study. You will be able to keep the money you have earned up to that point.

The results of your blood tests, the genotyping and your child's cognitive tests will be kept confidential.

In any sort of report we make public, we will not include any information that will make it possible to identify you. Research records will be kept in a locked file; only the researchers will have access to the records. We will need to videotape the infant's face in order to analyze reaction times based on the infant's eye movements. Only the researchers will have access to the videotape. Upon the conclusion of the data analysis, the videotape will be destroyed (by September 2015).

If you have questions: The main researcher conducting this study is Professor Marie Caudill. Please ask any questions you have now. If you have questions later, you may contact Marie Caudill at mac379@cornell.edu or at 607-254-7456. Should you contact Professor Caudill via email, please be aware that there is a chance that your answers could be read by a third party as internet and email transmissions are neither private or secure.

If you have any questions or concerns regarding your rights as a subject in this study, you may contact the Institutional Review Board (IRB) for human study participants at 607-255-5138 or access their website at http://www.irb.cornell.edu. You may also report your concerns or complaints anonymously through Ethicspoint or by calling toll free at 1-866-293-3077. Ethicspoint is an independent organization that serves as a liaison between the University and the person bringing the complaint so that anonymity can be ensured.

You will be given a copy of this form to keep for your records.

Statement of Consent: I have read the above information, and have received answers to any questions I asked. I consent to take part in the study.

Your Signature _____ Date _____

Study Representative Signature _____ Date_____

In addition to agreeing to participate, I also consent to having my child's cognitive function assessed and to having these activities videotaped during the session.

Your Signature _____ Date _____

Study Representative Signature _____ Date _____

This consent form will be kept by the researcher for at least five years beyond the end of the study and was approved by University of Cornell's Institutional Review Board for Use of Human Study Participants on June 5 2008 and by Cayuga's Medical Center's Review Board for Use of Human Study Participants on October 16, 2008.

Consent, Lactating Women

Cornell University
Informed Consent for Research Involving Human Study Participants

You are being invited to participate in a research study. This form is designed to provide you with information about this study. The Principal Investigator or representative will describe this study to you and answer any of your questions.

Project Title:

Effect of maternal choline intake on maternal/fetal biomarkers of choline status

Investigators:

Marie Caudill, PhD, RD; Associate Professor, Division of Nutritional Sciences, Cornell University

Eva Pressman, MD. Director of Obstetrics and Maternal-Fetal Medicine, Department of Obstetrics and Gynecology, University of Rochester School of Medicine, Rochester, New York

Richard Canfield, PhD. Research Associate, Division of Nutritional Sciences, Cornell University

Barbara Strupp, PhD. Professor, Division of Nutritional Sciences, Cornell University

Srisatish Devapatla, MD. Director, Special Care Nursery, Chairman- Dept. of Pediatrics, Cayuga Medical Center at Ithaca, Ithaca, New York.

Sandy Saintonge, MD. Assistant Professor of Clinical Public Health and Clinical Pediatrics, Weill Cornell Medical College, Attending Physician, New York Hospital Queens, Department of Emergency Medicine

Cydne Perry, PhD. Postdoctoral Research Associate, Division of Nutritonal Sciences, Cornell University

What the study is about:

Choline, a nutrient found in the diet (i.e., eggs, milk, and beef), was recently recognized as a required nutrient for humans. However, at present, the amount of choline required during pregnancy and lactation is unknown. Current dietary intakes in the general population range from ~ 100 to 1000 mg/d with an average intake of 300 mg/d. In 1998, an "Adequate Intake" level for choline of 425, 450 and 550 mg/d was established for non-pregnant, pregnant and lactating women respectively.

However, these estimates probably exceed actual requirements given that habitual choline intake levels in healthy populations are ~ 300 mg/d.

There is evidence from animal studies that extra choline consumed by the mother during and after pregnancy may have long lasting beneficial effects on the memory and attention of the newborn. However, no study has examined this in humans.

Thus, this study seeks to examine choline metabolism (i.e., use) and requirements in pregnant and lactating women. In addition, it seeks to assess whether higher choline intakes improve biomarkers of choline status (i.e., increase blood concentrations).

Finally, there is evidence that choline status affects folate (another essential nutrient) and vice versa. *Thus, this study seeks to examine the relationship between choline and folate status.*

What we will ask you to do:

Screening Phase:

First, it is necessary to determine whether you meet the inclusion criteria of this study. This process will take up to 1 hour. The inclusion criteria are as follows:

- I am a lactating mom who plans to exclusively breast feed for the duration of the study (ie, the next 10 weeks).
- I can begin the feeding study at the start of my fifth week of lactation.
- I am between 21 and 40 years of age.
- I eat meat and other animal products.
- I am not a current smoker.
- I am not using recreational drugs.
- I am not drinking alcoholic beverages.
- I am healthy.
- I do not have kidney or liver problems.
- I am not chronically taking medication that affects liver function.

During the screening phase, you will have your blood drawn (~ 4 tablespoons) which will be used to assess your health status and measure select vitamins/nutrients. DNA will also be obtained from your blood in order to investigate the effect of nutrients for people of different genetic make-up. You will also complete a health history questionnaire.

Dietary Intervention Phase

If you meet the required criteria, you will be invited to participate in a ten week feeding study. Throughout this study you will consume a normal mixed diet consisting of breakfast, lunch, dinner and snacks. You will consume at least one meal every Monday, Wednesday, and Friday (breakfast, lunch or dinner) in the human metabolic research unit located at Cornell University. The other meals will be provided as takeout meals. You will also consume a prenatal vitamin/mineral supplement to ensure that you are meeting the recommended dietary intake levels for lactating women of all the essential nutrients.

The study diet provides 350 mg/d choline and contains food items that are significant sources of choline (i.e., eggs, beef, and milk). In addition, you will be randomized to a choline supplement to achieve total choline intakes of 450 or 900 mg/d. To increase the study's validity, the level of choline intake to which you are assigned (i.e., 450 or 900 mg/d) will not be disclosed.

To help us better understand choline requirements and use during lactation, a small portion of the choline will be labeled from study week 6 to 10. This label (marker) is safe and has been used many times in studies involving babies, young children and pregnant / lactating women.

It is important that you eat all of the food that we give you and that you eat nothing else outside of the study food. You will be weighed every week and extra food/beverages will be provided as necessary to ensure that calorie needs are being met.

Blood will be taken by a trained phlebotomist (i.e., a person that is trained to draw blood) before the start of the study and at weeks 3, 6, 9, and 10. The blood will be used to analyze how choline intake, folate intake and/or lactation influences biomarkers of status. Also we will use the DNA obtained from the blood to analyze how genetic differences influence the use of choline and/or folate.

In addition, you will be required to collect your urine over a 24 hour period and to provide a breast milk sample at the beginning of the study (week 0) and at weeks 3, 6, 9 and 10. The breast milk will be expressed completely from one breast (either right or left) at the beginning of the second feeding time (ie, 10 am). All supplies/materials needed for this expression will be provided. Samples obtained during your participation on this study may be used by the investigators (or collaborators) for the purposes of learning more about other nutrients (ie., vitamin D and biotin) and how genetic differences affect requirements for these nutrients.

It is possible that other investigators will have access to your blood samples (including DNA) and urine samples; however, the samples will be de-identified (ie, no names will be used).

Cognitive Testing of Infant

We would like to perform cognitive tests that assess your infant's attention and memory when he/she is three, six, nine and twelve months of age. These tests will measure your child's eye movements to changes in pictures/images. Feeding your baby between tests is fine. These tests are non-invasive. Each test is approximately 5-10 minutes and will take up to 30 minutes total.

Risks and benefits:

We foresee minimal risks as part of your participation in this study. The choline dose of 450 mg/d is below the "adequate intake" level of 550 mg/d; however, it is above the average intake level of 300 mg/d consumed by the general population. In addition, the 450 mg/d choline intake level in this study is comprised of dietary choline obtained from a normal mixed diet (i.e., 350 mg/d) and supplemental choline (100 mg/d). **The choline dosage of 900 mg/d is considered to be safe (Institute of Medicine, National Academy of Science) and falls within the upper range of choline intake consumed by the general population.** ***None-the-less, we will monitor your health status as you progress through this study. Specifically we will obtain a blood chemistry profile and a complete blood count at weeks 0, 3, 6, 9 and 10. These tests provide information on your blood lipids, liver and kidney function, and iron status. We will also take your blood pressure on a weekly basis. Should any problems be identified, we will refer you to your physician who will advise on your continuing to participate in this study.***

As with any blood draw, it is also possible that you will experience some dizziness, ill-feeling and bruising during/after the blood draw. In addition, having blood samples taken always includes a certain low risk of infection. ***To minimize this risk, blood will be drawn by a trained phlebotomist at the human metabolic research unit while you are lying down.***

There are no established direct benefits to you or your baby. From this study we hope to draw conclusions regarding the optimal intake levels of choline and folate in lactating women, potentially benefiting the health of other women and children.

Compensation:

Week of Study	Payment
1	$50
2	$60
3	$70
4	$80
5	$100
6	$120
7	$140
8	$160
9	$200
10	$220

You will be paid the dollar amounts indicated above in two allotments ($360 at week 5 and $840 at week 10) for a total of $1200. Should you stop participating in the study, you will be compensated for the time you have spent on the study.

You will also be compensated for each cognitive evaluation session ($50 per session x 4 sessions for a total of $200).

Taking part is voluntary:

Your participation in this study is completely voluntary. If you decide to take part, you are free to withdraw at any time; however you will not be eligible to receive monetary compensation after ending the study. You will be able to keep the money you have earned up to that point.

The results of your blood tests, the genotyping and your child's cognitive tests will be kept confidential.

In any sort of report we make public, we will not include any information that will make it possible to identify you. Research records will be kept in a locked file; only the researchers will have access to the records. We will need to videotape the infant's face in order to analyze reaction times based on the infant's eye movements. Only the researchers will have access to the videotape. Upon the conclusion of the data analysis, the videotape will be destroyed (by September 2015).

If you have questions: The main researcher conducting this study is Professor Marie Caudill. Please ask any questions you have now. If you

have questions later, you may contact Marie Caudill at mac379@cornell.edu or at 607-254-7456. Should you contact Professor Caudill via email, please be aware that there is a chance that your answers could be read by a third party as internet and email transmissions are neither private or secure.

If you have any questions or concerns regarding your rights as a subject in this study, you may contact the Institutional Review Board (IRB) for human study participants at 607-255-5138 or access their website at http://www.irb.cornell.edu. You may also report your concerns or complaints anonymously through Ethicspoint or by calling toll free at 1-866-293-3077. Ethicspoint is an independent organization that serves as a liaison between the University and the person bringing the complaint so that anonymity can be ensured.

You will be given a copy of this form to keep for your records.

Statement of Consent: I have read the above information, and have received answers to any questions I asked. I consent to take part in the study.

Your Signature _____ Date _____

Study Representative Signature _____ Date _____

In addition to agreeing to participate, I also consent to having my child's cognitive function assessed and to having these activities videotaped during the session.

Your Signature _____ Date _____

Study Representative Signature _____ Date _____

This consent form will be kept by the researcher for at least five years beyond the end of the study and was approved by University of Cornell's Institutional Review Board for Use of Human Study Participants on October 15, 2008.

Consent, Nonpregnant Women

Cornell University
Informed Consent for Research Involving Human Study Participants

You are being invited to participate in a research study. This form is designed to provide you with information about this study. The Principal Investigator or representative will describe this study to you and answer any of your questions.

Project Title:

Effect of maternal choline intake on maternal/fetal biomarkers of choline status

Investigators:

Marie Caudill, PhD, RD; Associate Professor, Division of Nutritional Sciences, Cornell University

Eva Pressman, MD. Director of Obstetrics and Maternal-Fetal Medicine, Department of Obstetrics and Gynecology, University of Rochester School of Medicine, Rochester, New York

Richard Canfield, PhD. Research Associate, Division of Nutritional Sciences, Cornell University

Barbara Strupp, PhD. Professor, Division of Nutritional Sciences, Cornell University

Srisatish Devapatla, MD. Director, Special Care Nursery, Chairman- Dept. of Pediatrics, Cayuga Medical Center at Ithaca, Ithaca, New York

Sandy Saintonge, MD. Assistant Professor of Clinical Public Health and Clinical Pediatrics, Weill Cornell Medical College, Attending Physician, New York Hospital Queens, Department of Emergency Medicine

Cydne Perry, PhD. Postdoctoral Research Associate, Division of Nutritonal Sciences, Cornell University

What the study is about:

This study seeks to provide data about nutrient requirements / utilization during pregnancy (see below). *You are being invited for possible participation in this study in order to examine choline requirements/use in non-pregnant women and to serve as a control for the pregnant women.*

Choline, a nutrient found in the diet, was recently recognized as a required nutrient for humans. The recommended dietary intake level for non-

pregnant and pregnant women is 425 or 450 mg/d, respectively. However, at present, it is not known whether 450 mg/d is enough to maintain choline status in pregnant women. *Thus, this study seeks to assess the effect of pregnancy on choline status and to examine whether the current recommended dietary intake, 450 mg/d, is enough to maintain choline status during pregnancy. Your participation in this study will enable us to compare your choline status to that of a pregnant woman and in doing so assess the effect of pregnancy on choline status.*

It is possible that choline affects the use of folate, another essential nutrient and vice versa. *Thus, this study seeks to examine the relationship between choline and folate.*

What we will ask you to do:

Screening Phase:

First, it is necessary to determine whether you meet the inclusion criteria of this study. This process will take up to 1 hour. The inclusion criteria are as follows:

- I am not pregnant or lactating
- I am between 21 and 40 years of age
- I eat meat and other animal products
- I am not a current smoker.
- I am not using recreational drugs
- I am willing to refrain from drinking alcoholic beverages during the 12-week study period
- I am healthy
- I do not have anemia.
- I do not have kidney or liver problems.
- I am not chronically taking medication that affects liver function.
- I have a body mass index (BMI) $\leq$ 34

During the screening phase, you will have your blood drawn (~ 4 tablespoons) which will be used to assess your health status and measure select vitamins/nutrients. DNA will also be obtained from your blood in order to investigate the effect of nutrients for people of different genetic make-up. You will also complete a health history questionnaire.

Study Phase

If you meet the required criteria, you will be invited to participate in a twelve week feeding study. Throughout this study you will consume a normal mixed diet consisting of breakfast, lunch, dinner and snacks. You will consume at least one meal per day (breakfast or dinner) in the human metabolic research unit located at Cornell University. The other meals will be provided as takeout meals. You will also consume a customized vitamin/mineral supplement to ensure that you are meeting the recommended dietary levels for pregnant (and non-pregnant) women of all the essential nutrients. In addition, you will be randomized to a

choline supplement to acheive total choline intakes of 450 or 900 mg/d. To increase the study's validity, the level of choline intake to which you are assigned (i.e., 450 or 900 mg/d) will not be disclosed.

To help us better understand choline requirements and use during pregnancy, a small portion of the choline or other relevant nutrients will be labeled from week 6 to 12. This label (marker) is safe and has been used many times in studies involving babies, young children and pregnant women.

It is important that you eat all of the food we give you and that you eat nothing outside what is provided. You will be weighed every week and your calorie intake will be modified to ensure that you do not lose or gain more than a couple of pounds.

Blood will be taken by a trained phlebotomist before the start of the study and at weeks 3, 6, 9, 10 and 12. The blood will be used to analyze how choline intake, folate intake and/or pregnancy influenced biomarkers of status. Also we will use the DNA obtained from the blood to analyze how changes in genes influence the use of choline and/or folate. In addition, you will be required to collect your urine over a 24 hour period at the beginning of the study (week 0) and at weeks 6, 9, and 12. Samples obtained during your participation on this study may be used by the investigators (or collaborators) for the purposes of learning more about other nutrients (ie., vitamin D and biotin) and how genetic differences affect requirements for these nutrients.

It is possible that other investigators will have access to your blood (including DNA) and urine samples; however, the samples will be de-identified (ie, no names will be used).

Risks and benefits:

We foresee minimal risks as part of your participation in this study. All essential nutrients will be provided at or above the recommended intake level for non-pregnant women. The choline dosage of 900 mg/d is considered safe (Institute of Medicine, National Academy of Science) and falls within the range of choline intake consumed by the general population.

It is possible that you will experience some dizziness or ill-feeling during the blood draw. In addition, having blood samples taken always includes

a certain low risk of infection. *To minimize this risk, blood will be drawn by a trained phlebotomist at the human metabolic research unit while you are lying down.*

There are no established direct benefits to you. From this study we hope to draw conclusions regarding the optimal intake levels of choline and folate for pregnant women, potentially benefiting the health of other women and children.

In the event that you should be injured in the course of this research study, you will be provided with necessary medical care in Cayuga Medical Center At Ithaca. This statement does not mean that either such medical care or hospitalization, if necessary, will be free of charge.

Compensation:

Week of Study	Payment
1	$50
2	$60
3	$70
4	$80
5	$100
6	$120
7	$140
8	$160
9	$180
10	$200
11	$220
12	$240

You will be paid the dollar amounts indicated above in two allotments ($480 at week 6 and $1140 at week 12) for a total of $1620. Should you stop participating in the study, you will be compensated for the time you have spent on the study.

Taking part is voluntary:
Participating in this study is completely voluntary. If you decide to take part, you are free to withdraw at any time; however you will not be eligible to receive monetary compensation after ending the study. You will be able to keep the money you have earned up to that point.

The results of your blood tests including the genotyping will be kept confidential.

In any sort of report we make public, we will not include any information that will make it possible to identify you. Research records that contain

your name (i.e., informed consent, health history questionnaire, and the blood chemistry profiles and complete blood counts) will be kept in a locked filing cabinet in the office of the PI. All other data (ie., blood measurements, DNA sequences) will be identified only by a code (i.e., number).

If you have questions: The main researcher conducting this study is Professor Marie Caudill. Please ask any questions you have now. If you have questions later, you may contact Marie Caudill at mac379@cornell.edu or at 607-254-7456. Should you contact Professor Caudill via email, please be aware that there is a chance that your answers could be read by a third party as internet and email transmissions are neither private or secure.

If you have any questions or concerns regarding your rights as a subject in this study, you may contact the Institutional Review Board (IRB) at 607-255-5138 or access their website at http://www.irb.cornell.edu. You may also report your concerns or complaints anonymously through Ethicspoint or by calling toll free at 1-866-293-3077. Ethicspoint is an independent organization that serves as a liaison between the University and the person bringing the complaint so that anonymity can be ensured.

You will be given a copy of this form to keep for your records.

Statement of Consent: I have read the above information, and have received answers to any questions I asked. I consent to take part in the study.

Your Signature _____ Date _____

Study Representative Signature _____ Date _____

This consent form will be kept by the researcher for at least five years beyond the end of the study and was approved by University of Cornell's Institutional Review Board for Use of Human Study Participants on June 5 2008 and by Cayuga Medical Center's Review Board for Use of Human Study Participants on October 16 2008.

Study Diet: Seven-day menu consumed by pregnant, lactating, and nonpregnant study participants[1]

Day	Breakfast	Lunch	Dinner	Folate content[2]
Monday	Scrambled eggs (2 large) WW[3] toast (2 slices) Peaches (1 snack cup)	Pesto sandwich: WW[3] bread (2 slices) Pesto (15 g) Swiss cheese (42 g) Romaine lettuce (20 g) Celery sticks (30 g) Carrot sticks (30 g)	Beef and cheese tacos: Hard corn tortillas (3) Ground beef (80% lean; 105 g) Cheddar cheese (30 g) Sour cream (60 g) Salsa (90 g) Iceberg lettuce (20 g) Melon (112 g) Milk (10 oz)	Breakfast- 133 µg Lunch- 101 µg Dinner- 125 µg Snack- 56 µg ***Total***- 415 µg
Tuesday	Unenriched waffle (1) Hardboiled egg (1 large)	Tuna sandwich: WW[3] bread (2 slices) Tuna, canned (56 g) Cheddar cheese (30 g) Iceberg lettuce (30 g) Mayonnaise (15 g) Grapes (100 g)	Spaghetti: Unenriched pasta (220 g) Tomato sauce (220 g) Mushrooms (30 g) Parmesan cheese (10 g) Mozzarella cheese (25 g) Milk (10 oz)	Breakfast- 48 µg Lunch- 116 µg Dinner- 89 µg Snack- 56 µg ***Total***- 308 µg
Wednesday	Wegman's Fitness Crunch Cereal (80 g) Milk (10 oz) Raisins (1 box) Banana (1 medium)	Pastrami sandwich: WW[3] bread (2 slices) Pastrami (24 g) Swiss Cheese (23 g) Romaine lettuce (30 g) Cucumber slices (80 g)	Vegetarian pizza: Unenriched dough (200 g) Tomato sauce (112 g) Roasted red peppers (60 g) Mushrooms (30 g) Spinach (20 g)	Breakfast- 134 µg Lunch- 103 µg Dinner- 182 µg Snack- 56 µg ***Total***- 475 µg

			Mozzarella cheese (50 g) Apple sauce (1 snack cup) Milk (10 oz)	
Thursday	Unenriched blueberry pancakes (2) Blueberries (25 g/pancake)	Egg salad sandwich: WW[3] bread (2 slices) Hardboiled egg (1 large) Mayonnaise (20 g) Romaine lettuce (45 g) Celery sticks (30 g) Carrot sticks (30 g)	Beef and broccoli stir-fry: Steak beef (160 g) Broccoli (110 g) Un-enriched rice (200g) Onions (13 g) Melon (112 g) Milk (10 oz)	Breakfast- 30 µg Lunch- 130 µg Dinner- 353 µg Snack- 56 µg ***Total***- 569 µg
Friday	WW[3] bagel (1) Mandarin oranges (1 snack cup)	Bean burrito: WW[3] flour tortilla (1) Black beans (40 g) Cheddar cheese (30 g) Un-enriched rice (30 g) Salsa (60 g) Sour cream (20 g)	Lasagna: WW[3] lasagna noodles (2) Tomato sauce (150 g) Ground beef (80% lean; 60 g) Cottage cheese (40 g) Mozzarella cheese (10 g) Parmesan cheese (10 g) Summer squash (40 g) Zucchini (40 g) Milk (10 oz)	Breakfast- 74 µg Lunch- 46 µg Dinner- 122 µg Snack- 56 µg ***Total***- 298 µg
Saturday	Un-enriched raspberry muffins (2) Banana (1 medium)	Turkey sandwich: WW[3] bread (2 slices) Turkey (25 g) Provolone cheese (23 g)	Chicken quesadilla: WW[3] flour tortillas (2) Chicken (65 g) Cheddar cheese (150 g)	Breakfast- 68 µg Lunch- 62 µg Dinner- 201 µg Snack- 56 µg

		Iceberg lettuce (20 g) Cucumber slices (80 g)	Corn (100 g) Milk (10 oz)	***Total***-	387 µg
Sunday	Granola cereal (80 g) Milk (10 oz) Raisins (1 box) Peaches (1 snack cup)	Vegetable soup cup (1) Un-enriched corn muffin (1) Grapes (100 g)	Goulash: Un-enriched pasta (200 g) Canned tomatoes (50 g) Tomato puree (100 g) Canned potatoes (35 g) Onion (30 g) Steak beef (120 g) Canned pineapple (70 g) Milk (10 oz)	Breakfast- Lunch- Dinner- Snack- ***Total***-	47 µg 109 µg 162 µg 56 µg 374 µg

[1] Dietary folate was measured after trienzyme digestion with the microbiological assay as described in chapter 1

[2] Daily snack included a low fat yogurt (vanilla, raspberry, or peach; 170 g) and a low-sodium V8 (5.5 oz)

[3] WW, whole wheat

APPENDIX C: Publication authorizations

Folate status response to a controlled folate intake among nonpregnant, pregnant, and lactating women

A version of this manuscript was accepted for publication by *The American Journal of Clinical Nutrition* in June 2012 with the following authorship: Allyson A. West, Jian Yan, Cydne A. Perry, Xinyin Jiang, Olga V. Malysheva, and Marie A. Caudill, all of the Division of Nutritional Science at Cornell University. *The American Journal of Clinical Nutrition* Authors' Statement and Copyright Release Form (http://www.ajcn.org/site/misc/Authors%27_Agreement_Form.pdf) authorizes the inclusion of the manuscript in this dissertation in the following section:

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Genetic variation: impact on folate (and choline) bioefficacy

A version of this manuscript was published in the *International Journal for Vitamin and Nutrition Research*, 2010; 80:319-29, with the following authorship: Allyson A. West and Marie A. Caudill, both of the Division of Nutritional Science at Cornell University. E-mail correspondence with the editor of this journal shown below authorizes the inclusion of the manuscript in this dissertation.